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SECTION MEETINGS

ILLINOIS	
University of Chicago	December 9, 1947
MINNESOTA	
University of Minnesota	December 10, 1947
NEW YORK	
New York Academy of Medicine	December 10, 1947
PACIFIC COAST	
University of California	December 10, 1947
SOUTHWESTERN	
University of Oklahoma	November 15, 1947
WISCONSIN	
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16185

Cultivation of Toxoplasma in the Developing Chick Embryo.*

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Reports of the cultivation of toxoplasma in the developing chick embryo have appeared in the literature but these accounts are brief and give little experimental data. Levaditi, *et al.*,[1] and Wolfe, Cowen, and Paige[2] stated that inoculated fertile eggs hatched but that the chicks died within several days and their tissues were parasitized. Weinman[3] found that numerous small white nodules developed on the chorio-allantoic membrane after chorio-allantoic or yolk sac inoculation. In the above investigations recently isolated strains of toxoplasma were used for egg inoculation; deaths appeared to have been infrequent for they were not mentioned specifically. The data to be presented demonstrate that a strain of human origin, well adapted to mice, not only readily infected eggs but also produced such uniform embryo deaths as to make the embryonated egg a satisfactory titration medium.

The "R.H." strain of toxoplasma isolated by Sabin[4] from a fatal human case of encephalitis was used in these studies. It was

* This study was supported by a grant-in-aid from the United States Public Health Service.

1 Levaditi, C., Sanchis-Bayarri, V., Lepine, P., and Schoen, R., *Ann. de l'Inst. Pasteur,* 1929, **43**, 673.

2 Wolfe, A., Cowen, D., and Paige, B. H., *J. Exp. Med.*, 1940, **71**, 187.

3 Weinman, D., *Puerto Rico J. Pub. Health and Trop. Med.*, 1944, **20**, 125.

4 Sabin, A. B., *J. A. M. A.*, 1939, **116**, 801.

maintained in [illegible]ory by continuous intracerebral [illegible]ice and the 304th to 344th pass[illegible] was used for initiating the c[illegible]ryonated eggs. Fertile hen's egg[illegible]n-tested flocks were inocul[illegible]ted [illegible]ac. Incubation of the egg[illegible]ot [illegible]fter inoculation was at 37. I[illegible]50 [illegible]mice and chick embryos [illegible]cu[illegible]ccording to the method o[illegible]d [illegible]nch.[5]

Injectio[illegible]opla[illegible]ma-infected, mouse brain susp[illegible]to the yolk sac of embryonated [illegible]t least 6 different occasions led [illegible]ication of the parasites with resultin[illegible]the embryo in 4 to 10 days after [illegible]. Examination of the dead embryo[illegible]d the presence of numerous yello[illegible]ite plaques, 0.5 to 3 mm in diameter, on the chorio-allantoic and amniotic membranes (Fig. 1). The areas surrounding the lesions were thickened and histologically represented regions of dense cellular infiltration containing numerous toxoplasma (Fig. 2). Smears of the chorio-allantoic membrane and the yolk sac stained with Wright's stain revealed numerous toxoplasma both free and intracellular.

Three series of egg passages were studied in order to seek evidence of adaptation or change.

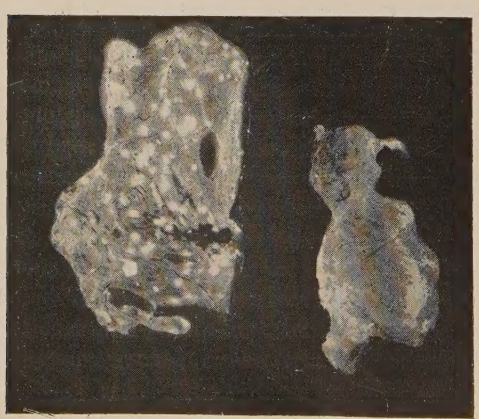

FIG. 1.
Toxoplasma in the developing chick embryo. Eggs 18 days old.
Left. Infected chorio-allantoic membrane.
Right. Normal chorio-allantoic membrane.

[5] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

One series was carried through 27 consecutive passages in 10- or 11-day-old fertile eggs (Table I). A second series was carried through 5 passages in 9- or 10-day incubated eggs, and three egg passages were done in which 6- or 7-day incubated eggs were utilized. No evidence was obtained that continuous or multiple egg passages altered the length of time between inoculation and death of the embryo. Most of the deaths occurred between the 5th and 6th days. Titrations performed in mice showed that LD_{50} titers for the various egg passages were about the same. Titrations of the various egg tissues revealed that while the chorio-allantoic membranes contained the greatest number of parasites the yolk sacs had only slightly less. Organisms were present in small number in the embryonic fluids (LD_{50} titers of $10^{-2.0}$ to $10^{-3.0}$) and the viscera of embryos (stained smears). Membranes harvested either from dead or living chick embryos at the time of the maximum number of embryo deaths possessed high titers (LD_{50} titers of $10^{-4.5}$ to $10^{-5.0}$) while the membranes of living eggs harvested two days before this maximum time gave lower titers ($10^{-3.5}$).

Although toxoplasma multiplied well in fertile hen's eggs between 6 and 11 days of age deaths in the younger embryos were spread over a larger number of days, *i.e.*, 4 to 8 days.

Several comparative titrations were performed in mice and chick embryos and the results are listed in Table II. In 3 of the tests higher LD_{50} titers were obtained in chick embryos. In these cases the amount injected into eggs was proportionately larger. However when similar inocula were used, *i.e.*, 0.5 ml per egg via the yolk sac and 0.5 ml per mouse intraperitoneally, the LD_{50} titers were identical. When the actual number of LD_{50} doses per gram of tissue were calculated the values obtained in three of the tests were slightly higher in mice than in chick embryos.

Other studies revealed that storage of infected membranes at 4°C yielded viable organisms for periods up to one month. Attempts to cultivate toxoplasma in the pc[illegible] embryonic fluids from 17-day fertile eggs

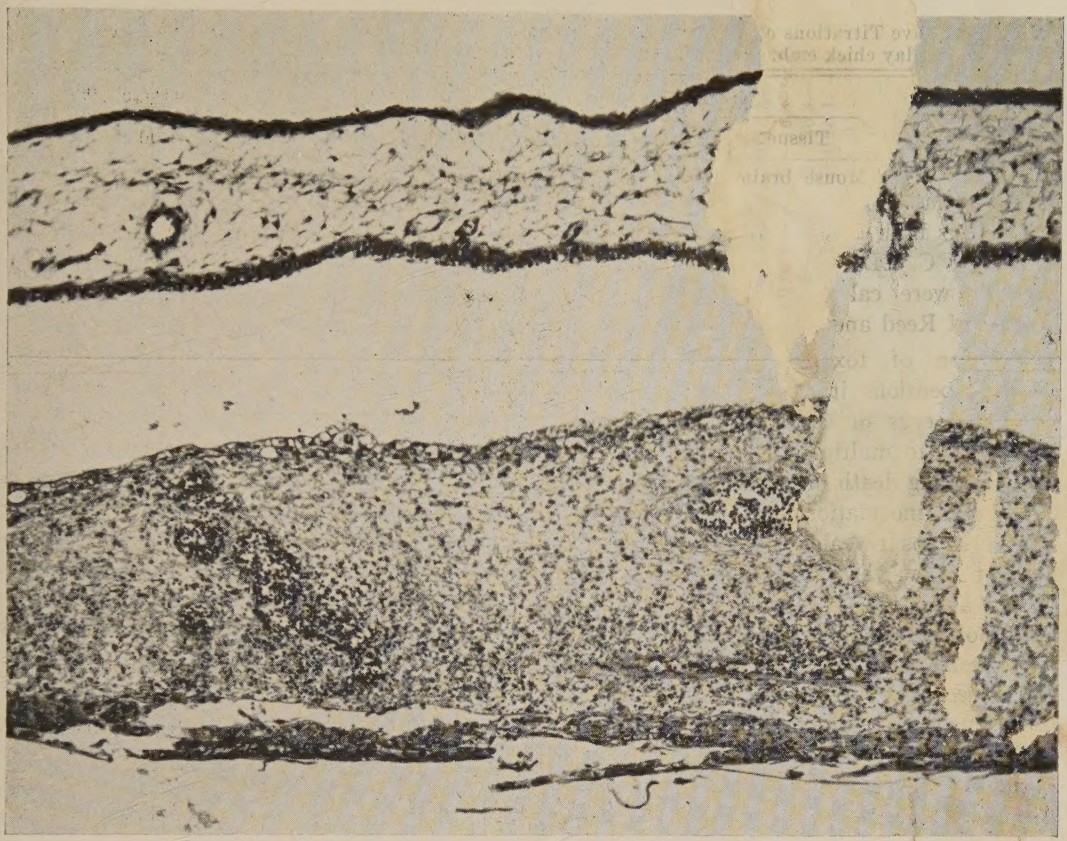

FIG. 2.
Toxoplasma in the developing chick embryo. Eggs 18 days old. H. & E. ×160.
Upper. Normal chorio-allantoic membrane.
Lower. Infected chorio-allantoic membrane.

TABLE I.
Toxoplasma Passages in Eggs Inoculated via the Yolk Sac.
0.5 ml per egg in 10- and 11-day-old embryonated hen's eggs.

Egg passage	Tissue suspension employed	Day of death	Intracerebral LD_{50} titers in mice		
			Yolk sac	Chorio-allantoic membrane	Membrane pool*
1	10% mouse brain	5,5,6,6,6,6,7,7,7,7	10-3.5	10-4.0	
4	5% Y.S.-C.A.*	6,6,6,6,6			10-4.5
7	10% ,, ,,	5,6,6,6			10-5.5
10	,, ,, ,,	4,5,6,6,6,6,6,6,6			10-5.0
12	,, ,, ,,	4,4,5,5,5,5,5,5			10-4.5
15†	,, ,, ,,	6,6,6,10			10-4.0
20	,, ,, ,,	5,5,5,5,6,6,6,6			10-5.0
27	,, ,, ,,	6,6,6,6,6	10-3.5	10-4.5	

* Pool of yolk sac and chorio-allantoic membrane.
† Inoculated with membranes which had been stored 5 days at °C.

unsuccessful. More than 13 passages through eggs did not result in any obvious modification of pathogenicity for animals. Inoculation of infected embryonal tissues caused disease in mice, rats, chicks, rabbits, and a rhesus monkey. Preliminary results indicated

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TABLE II.

Comparative Titrations of Toxoplasma Infected Tissues in Mice and Developing Chick Embryos. 10-day chick embryos inoculated via the yolk sac. Mice inoculated intracerebrally.

Passage	Tissue	Inoculation: Amt, ml	Inoculation: Titration in:	LD_{50} titer	LD_{50} doses per g of indicated tissue, × 1000
335	Mouse brain	.5	Embryos	$10^{-5.0}+$	200+
		.03	Mice	$10^{-4.5}$	1,000
336	" "	.5	Embryos	$10^{-5.0}$	200
		.03	Mice	$10^{-4.0}$	330
344	" "	.5	Embryos	$10^{-4.5}$	64
		.5*	Mice	$10^{-4.5}$	64
15	Y.S.-C.A.	.5	Embryos	$10^{-4.0}$	20
		.03	Mice	$10^{-3.0}$	33

* Mice in this case injected intraperitoneally.

that neutralization tests could be performed in the chick embryo.

The embryonated hen's egg may be an adjunct for the primary isolation of toxoplasma and although inoculation of eggs with the laboratory-adapted strain gave uniformly good results the probability is that for the initial recovery of strains the mouse is still the animal of choice.

Summary. A laboratory-adapted strain of toxoplasma was successfully propagated in the embryonated hen's egg. Uniform mortality was obtained and the majority of deaths occurred between 5 and 6 days after inoculation. The concentration of organisms in the chick embryo gave LD_{50} titers of $10^{-4.5}$ to $10^{-5.0}$ which approximated that usually attained in mice. Repeated passage through eggs resulted in no modification of pathogenicity for animals.

16186 P

Histochemical Differentiation Between Esterases.*

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Strikingly different properties of esterases (lipases) obtained from various species[1,2] have been reported by a number of workers. The differences include substrate specificity,[3] stereochemical specificity,[4] behavior towards activators and inhibitors, and pH optima.[5] Owing to multiple overlapping of differences and similarities between enzymes obtained from various sources, it is still undecided whether the various effects are due to the existence of several well defined individual enzymes or to the presence of unidentified accompanying substances.

With a histochemical method for the visualization of sites of lipase activity, similar differences between the esterases of various organs were found.

Experimental. Organs of 8 freshly killed white mice (5 males and 3 females) were fixed in chilled acetone, dehydrated, embedded

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

1 Rona, P., and Bach, E., *Bioch. Z.*, 1920, **11**, 166.

2 Gyotoku, K., *Bioch. Z.*, 1928, 1939.

3 Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, **59**, 183.

4 Rona, P., and Ammon, R., *Bioch. Z.*, 1927, **181**, 49.

5 Davidsohn, H., *Bioch. Z.*, 1913, **49**, 249.

and stained according to the techniques previously published.[6,7] In addition to the original substrates, a new one, G-9096-CJ (Atlas Powder Co., Wilmington, Del.), a stearic ester of modified mannitan, was used. This new substrate seems to be hydrolyzed more rapidly than the related Tweens, but the localization and the relative intensity of the reaction in various organs is the same with all substrates. Pieces of practically all organs (liver, kidney, spleen, lung, heart, stomach, intestine, pancreas, salivary glands, testis, uterus, ovary, urinary bladder, etc.) were included in a single paraffin block, and serial sections were incubated with the substrate to which the substances to be tested were added.

The following substances were selected for their known effects on esterases: cholate,[8,9] quinine,[10–13] arsanilate,[1,10,11,13] eserine,[4,14] pilocarpine,[4] urethane,[15] caprylate,[16] n-butyraldehyde,[17] acetophenone,[17] hexylresorcinol[18] and NaCl.[19]

Results. Since a strict quantitative evaluation of histochemical reactions of this type is impossible, increase or decrease in the in-

TABLE I.
Effect of Various Substances on Lipase (Esterase) Activity.

	Liver	Pancreas	Kidney	Intestine	Stomach	Testis: Interstitial cells	Testis: Spermatic elements	Lung
Quinine, .005 to .01 M	1—	2— to 3—	1— to 2—	3—	2— to 3—	0 to 1—	0 to 1—	0 to 3—
Arsanilate, .0002 M	3—	1—	3—	3—	1—	3—	to 1—	2— to 3—
Taurocholate, .02 M	2— to 3—	2+ to 3+	3—	3—	2+ to 3+	2—	2—	2— to 3—
Pilocarpine, .01 M	0	0	0 to 1—	0 to 1—	0 to 1—	0 to 1—	0 to 1—	0 to 1—
Urethane, .1 M	0	0	0	0	0	0	0	0
Eserine, .002 to .005 M	0 to 1—	0 to 1—	0 to 2—	0 to 2—	0 to 2—	0	0	0
Caprylate, .005 M	2— to 3—	1—	3—	2—	1—	2—	2—	2—
n-Butyraldehyde, .005 M	0	0	0	0	0	0	0	0
Acetophenone, .005 M	0	0	0	0	0	0	0	0
Hexylresorcinol, .0005 M	0	0	0	0	0	0	0	0
NaCl, 1.2 M	0	0	0	0	0	0	0	0

The symbol 3— stands for complete suppression of enzyme activity.

6 Gomori, G., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 362.

7 Gomori, G., *Arch. Path.*, 1946, **41**, 121.

8 Willstätter, R., and Memmen, F., *Z. physiol. Chem.*, 1924, **133**, 229.

9 Willstätter, R., and Memmen, F., *Z. physiol.* Chem., 1924, **133**, 247.

10 Rona, P., and Pavlovic, R., *Bioch. Z.*, 1922, **130**, 225.

11 Rona, P., and Pavlovic, R., *Bioch. Z.*, 1923, **134**, 108.

12 Rona, P., and Takata, M., *Bioch. Z.*, 1923, **134**, 118.

13 Rona, P., and Haas, H. E., *Bioch. Z.*, 1923, **141**, 222.

14 Mendel, B., and Rudney, H., *Bioch. J.*, 1943, **37**, 59.

15 Rona, P., and Lasnitzki, A., *Bioch. Z.*, 1925, **163**, 197.

16 Weber, H. H. R., and King, C. G. J., *J. Biol. Chem.*, 1935, **108**, 131.

17 Weinstein, S. S., and Wynne, A. M., *J. Biol. Chem.*, 1935-36, **112**, 649.

18 Glick, D., and King, C. G., *J. Biol. Chem.*, [illegible] **97**, 675.

19 [illegible]ck, D., *Nature*, 1941, **148**, 662.

tensity of the reaction will be indicated only by plus and minus signs, with the arbitrary degrees 1, 2 and 3. (Table I). The patte[illegible] of response was markedly uniform in [illegible]ern mals, with the exception of the [illegible] all ani- haved erratically toward[illegible]ng which be- bition, one case [illegible]as quinine (no inhi- 3 cases, [illegible]336 [illegible]se; slight to moderate inhibition, [illegible]; complete suppression of the reaction, 4 cases).

The histochemical results are in good agreement with previous findings on the *in vitro* behavior of hepatic and pancreatic lipase towards quinine and arsanilate and on the similarity between the pancreatic and gastric enzymes, both being activated by bile salts.[9] On the other hand, the effect of several substances, markedly active in test tube experiments, such as urethane, butyraldehyde, acetophenone, hexylresorcinol and NaCl, cannot be observed under the conditions of the histochemical experiment. An interesting new finding is the difference in sensitivity to arsanilate between the enzyme of the interstitial cells (testis) and that of the spermatic elements. Another curious observation was a marked diffusion of the reaction around the sites of activity in the pancreas and the stomach, but nowhere else, when cholate was added to the substrate. In some cases such large numbers of lead sulfide granules were embedded in the protecting collodion membrane and on its surface as to make the exact localization of the enzyme virtually impossible. This finding may indicate an increased diffusibility of pancreatic and gastric lipase in the presence of bile salts.

Summary. Differences in the behavior of esterases (lipases) from various sources towards activator and inhibitor substances, similar to those found previously in *in vitro* experiments, can be observed also in tissue sections stained for lipase.

16187

Biological Activity of Crystalline Procaine Penicillin *In vitro* and *In vivo*.*

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In a recent communication Salivar, Hedger, and Brown[1] described the preparation and chemical properties of crystalline procaine penicillin. The present report deals with the biological activity and with the absorption and excretion of this form of penicillin and of other relatively insoluble salts of penicillin.

Materials and Methods. Unless otherwise specified, crystalline procaine penicillin G and dihydro-F, prepared from crystalline sodium penicillins G and dihydro-F, respectively, were used throughout this study. The potencies of these preparations were determined by the Oxford cup plate method;[2] the per cent G, non-G penicillin by a modification of the N-ethyl piperidine method.[3] Sensitivity determinations were carried out by a quantitative broth dilution technic. The method of Tompsett, Schultz, and McDermott,[4] using *Strepto-*

* The authors wish to express their appreciation to Mr. C. J. Salivar, Mr. O. Sumner, Mr. W. Armstrong, and Dr. P. Regna for preparation of the samples used in this study. They are indebted also to Mr. F. H. Hedger for chemical analyses of the preparations and to Mrs. W. Reed and Mrs. D. Rinne for assistance in carrying out the many biological assays necessary for this study.

1 Salivar, C. J., Hedger, F. H., and Brown, E., *J. A. C. S.*, 1948, in press.

2 Schmidt, W. H., Ward, G. E., and Coghill, R. D., *J. Bact.*, 1945, **49**, 411.

3 *Federal Register*, April 4, 1947, v. **12(67)**, p. 2222, 141.5 (f).

4 Tompsett, R., Schultz, S., and McDermott, [illegible] *J. Bact.*, 1947, **53**, 581.

coccus hemolyticus as the test organism and crystalline sodium penicillin G as standard, was used for determination of the concentrations of penicillin in blood and urine. Mouse protection tests were carried out using *Streptococcus hemolyticus* and *Diplococcus pneumoniae* as the test organisms and the method described by Hobby *et al.*[5] in a recent communication.

Activity of Crystalline Procaine Penicillin In Vitro. Two preparations of procaine penicillin G, having potencies of 1066 and 1025 units per mg, and 2 preparations of procaine penicillin dihydro-F, having potencies of 1010 and 987 units per mg, were used.† Likewise 3 preparations containing mixtures of crystalline procaine penicillins G and dihydro-F as well as certain naturally occurring penicillin pigments were used.‡ These showed potencies of 930 u/mg, 975 u/mg, and 950 u/mg, respectively. The sensitivity of a variety of organisms to these penicillins was determined as follows:

Six-hour plain broth cultures of *Streptococcus hemolyticus* (strain C230Mv), *D. pneumoniae* (strain I/230), *Staphylococcus aureus* (strain H), *Bc. subtilis, Streptococcus viridans, E. coli,* and *A. aerogenes* were used throughout. Cultures were diluted with broth to a constant density immediately prior to use. A density equivalent to a MacFarland $BaSO_4$ No. 1 standard and allowing 78% transmission on a Photovolt Lumetron No. 400 was arbitrarily chosen as standard. For each gram negative organism tested a series of 9 tubes were set up containing 0.1, 0.15, 0.2, 0.25 . . . . 0.5 ml of broth containing 400 units of procaine penicillin per ml. In a few instances, a concentration of 800 units per ml was essential. For the more sensitive gram positive organisms, concentrations of 0.1 unit per ml were used. The total volume of each tube was adjusted to 0.5 ml with sterile broth and 0.5 ml of a 10^{-3} dilution of the standardized culture was then added to each. The final concentration of organisms was, therefore, about 150,000 per ml. Incubation was carried out at 37°C for a period of 24 hours. The sensitivity of an organism was accepted as the least amount of penicillin causing complete inhibition of growth, as evidenced by absence of gross turbidity, after 24 hours incubation.

Crystalline sodium penicillins X, G, dihydro-F, and K§ were tested simultaneously for their activity against the same group of organisms.

As shown in Table I, crystalline procaine penicillins G and dihydro-F are highly effective antibacterial agents *in vitro.* The activity of crystalline procaine penicillin G at times may differ quantitatively, however, from that of the crystalline sodium salt of penicillin G. Under the experimental conditions used in this study, all organisms tested were slightly more sensitive to crystalline sodium penicillin G than to crystalline procaine penicillin G. No differences were observed in the sensitivities of these organisms to the sodium salt of penicillin dihydro-F as

5 Hobby, G. L., Burkhart, B., and Hyman, B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 296.

† The theoretical potencies of crystalline procaine penicillins G and dihydro-F are calculated to be 1041 and 986 units per mg respectively, based on crystalline sodium penicillins G and dihydro-F at 1667 and 1600 units per mg, respectively. Each mg of crystalline procaine penicillins G and dihydro-F contains 0.42 and 0.43 mg procaine base, respectively.

‡ On crystallization of procaine penicillins G and dihydro-F from impure penicillin containing approximately 70 to 80% G and 20 to 30% dihydro-F, certain of the naturally occurring impurities are precipitated with the penicillins. Nevertheless the penicillins appear to be in the crystalline form.

§ The crystalline sodium salts of penicillin used were identical with the preparations used by one of the present investigators (G.L.H.) in a previous study and have been described in detail elsewhere.[5] The preparation of crystalline penicillin G had a potency of 1634 units per mg by the bioassay method and a *Bc. subtilis, Staphylococcus aureus* differential ratio of 1.0. The polariscopic assay of this preparation was 1635 units per mg. Ultraviolet absorption indicates 100% G. The crystalline penicillin K used showed a potency of 2182 units per mg and a differential ratio of 0.36; the crystalline penicillin X, a potency of 1069 units per mg, and a differential ratio of 1.39; the purified penicillin dihydro-F, a potency of 1675 units per mg and a differential ratio of 0.57.

TABLE I.
Comparative Activity of Crystalline Procaine and Sodium Penicillins *in Vitro*.

Organism	Sensitivity in units per ml						
	Procaine penicillins			Sodium penicillins			
	G	Dihydro-F	Mixed*	G	Dihydro-F	X	K
D. pneumoniae (I/230)	0.085	0.042	0.033	0.030	0.040	0.005	0.022
Strep. hemolyticus (C203Mv)	0.022	0.020	0.015	0.015	0.025	0.004	0.012
Strep. viridans	0.220	0.200	0.150	0.100	0.200	0.075	0.100
Staph. aureus (H)	0.100	0.050	0.047	0.050	0.050	0.060	0.040
Bc. subtilis	0.025	0.035	0.018	<0.005	0.035	0.005	0.025
A. aerogenes	110.000	120.000	53.000	40.000	160.000	80.000	>200.000
E. coli	160.000	160.000	87.000	60.000	200.000	40.000	>200.000

* Crystalline procaine penicillins G and dihydro-F mixed, with accompanying naturally-occurring impurities.

compared to its procaine salt. Furthermore, their sensitivities to crystalline sodium penicillin G and to mixtures of crystalline procaine penicillins G and dihydro-F, with accompanying impurities, were identical.

Procaine penicillin may be prepared by the interaction of procaine hydrochloride and sodium penicillin. Procaine hydrochloride in low concentration has in itself no bacteriostatic action against this group of test organisms. It is chemically derived from para-aminobenzoic acid, and has been shown by Woods and Fildes[6] and by others,[7-11] to be capable of inhibiting the *in vitro* and *in vivo* bacteriostatic action of sulfadiazine against *Streptococcus hemolyticus* and certain other organisms. The possibility that procaine at times may serve as an essential metabolite and thus alter the concentration of penicillin necessary for inhibition of growth was, therefore, suggested. Procaine hydrochloride, however, in concentrations of 0.02, 1.0, and 100 mcg per ml failed to alter the sensitivity of *Streptococcus hemolyticus* to crystalline sodium penicillins G or dihydro-F.

Activity of Crystalline Procaine Penicillin In Vivo. Preparations of crystalline procaine penicillins G and dihydro-F in oil, diluted to contain 1,000 units of penicillin per ml, were used throughout this study. In a few instances preparations containing mixtures of crystalline procaine penicillins G and dihydro-F, with certain of the accompanying impurities of partially purified penicillin, were used. Crystalline potassium penicillin G in oil and beeswax was also tested for comparison.

Fifteen-hour blood broth cultures of a highly virulent strain of Group A hemolytic streptococcus (strain C203Mv) and of pneumococcus type I (Strain I/230) were used throughout. Mice were infected by the intraperitoneal route with one cc of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions of culture. Treatment was carried out by the subcutaneous route, a single injection of penicillin being administered 2 hours after infection. All experiments were controlled with a series of untreated animals. In all instances the penicillins used were administered in sesame or peanut oil, a concentration of 1,000 units of penicillin per ml being used.

As shown in Table II, procaine penicillin is an effective chemotherapeutic agent against hemolytic streptococcal and pneumococcal infections. Three hundred units of procaine penicillin G, administered in a single injection 2 hours after infection, is adequate to protect approximately 70% of animals against 10 to 10,000 lethal doses of hemolytic streptococci or pneumococci. Similar protection against hemolytic streptococcal infections was o.

6 Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

7 Woods, D. D., and Fildes, P., *Chem. and Industry*, 1940, **59**, 133.

8 Boroff, D. A., Cooper, A., and Bullowa, J. G. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 182.

9 Casten, D., Fried, J. J., and Hallman, F. A., *Surg. Gynecol. Obstet.*, 1943, **76**, 726.

10 Kelteh, A. K., Baker, L. A., Krahl, M. E., and Clowes, G. H. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 533.

11 Legge, J. W., and Durie, E. B., *Med. J. Australia*, 1943, **29**, 561.

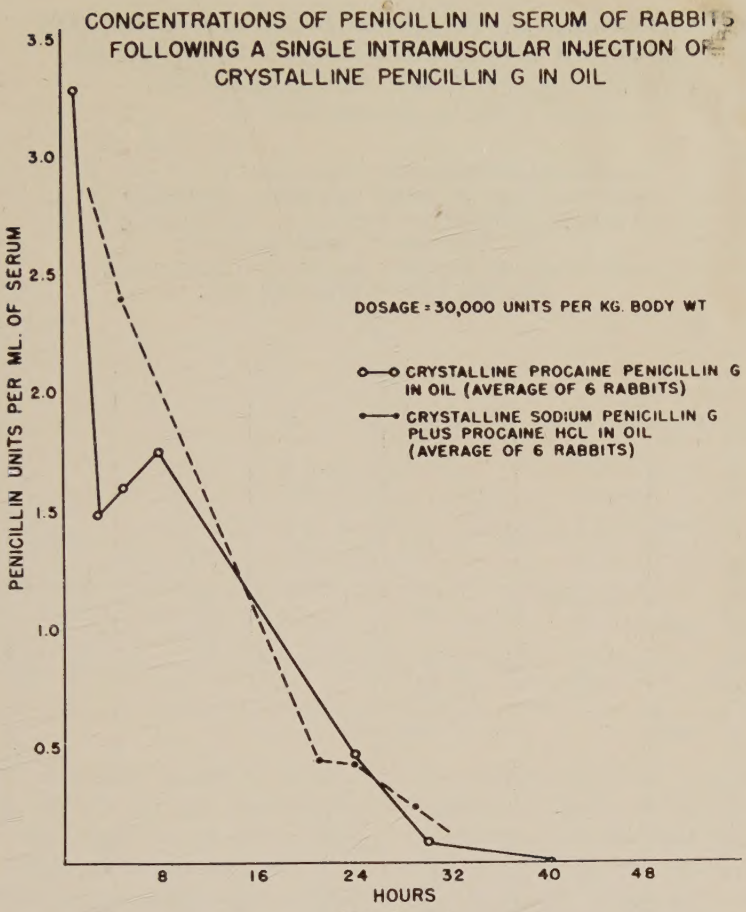

FIG. 1.

served with crystalline procaine penicillin dihydro-F.

The fact that certain impurities are capable of enhancing the action of crystalline sodium penicillin G has been discussed in previous communications.[5,12] Preparations containing mixtures of crystalline procaine penicillins G and dihydro-F with certain of the naturally occurring penicillin mold pigments or other impurities have shown a similar enhanced action. Whereas 300 units of crystalline procaine penicillin G were capable of protecting 70% of animals against infection due to *Streptococcus hemolyticus,* this amount of the mixed procaine penicillins G and dihydro-F, with accompanying impurities, was capable of protecting 90% of animals. One hundred and fifty units of these preparations of mixed crystalline penicillins were almost as effective as 300 units of crystalline procaine penicillin G or dihydro-F. The enhanced action of these mixed penicillins was not apparent in the small series of animals tested with pneumococcus.

Absorption and Excretion of Crystalline Procaine Penicillin. Crystalline procaine penicillins G and dihydro-F were prepared in a concentration of 300,000 units per ml peanut or sesame oil. Similar preparations of mixtures of crystalline procaine penicillins G and dihydro-F and accompanying impurities were also used. All experiments were carried out in normal male rabbits, weighing approximately 3 kg. In all instances 30,000 units per kg body weight were administered by the intra-

12 Hobby, G. L., Lenert, T. F., and Hyman, B., *J. Bact.*, 1947, **54**, 305.

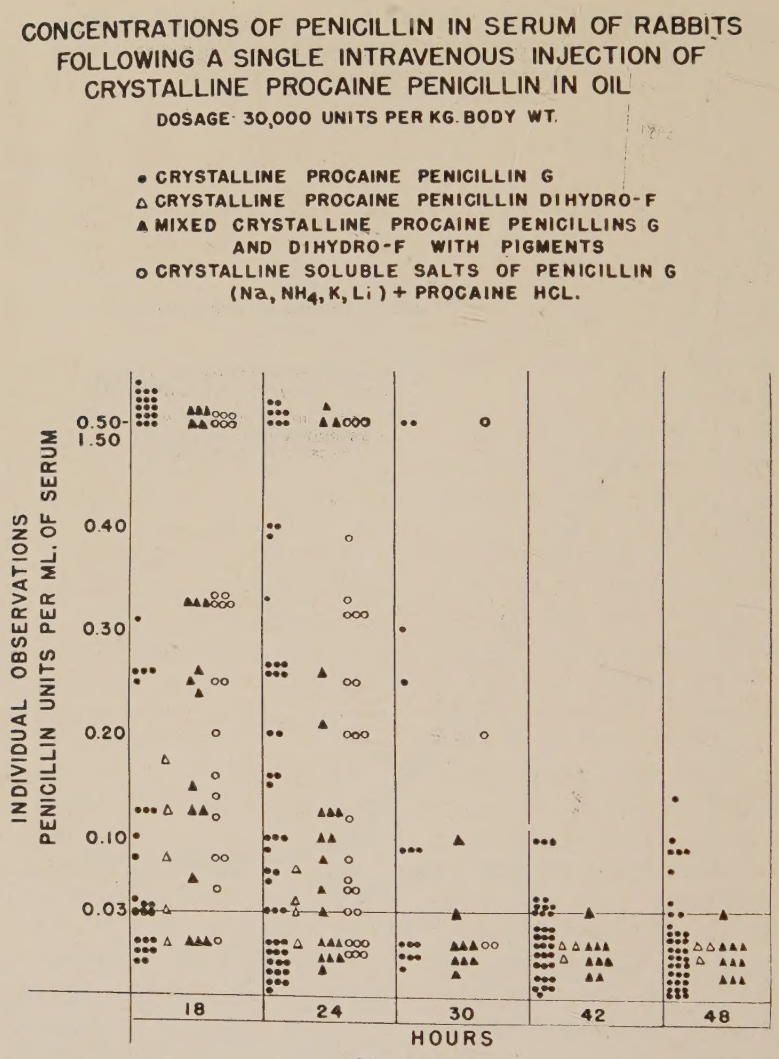

FIG. 2.

muscular route. Animals were bled immediately prior to and at varying intervals after injection. The concentration of penicillin in the blood was determined by the method of Tompsett, Schultz, and McDermott,[4] using undiluted serum as well as serum diluted 1:4 and 1:20. *Streptococcus hemolyticus* was used throughout as the test organism and crystalline sodium penicillin G as standard.‖ The minimum level detectable by this method varied from 0.02 to 0.04 units per ml. No attempt was made to determine the urinary excretion of penicillin from the rabbits injected.

As indicated in Fig. 1 and 2, a single intramuscular injection of 30,000 units of crystalline procaine penicillin G in oil per kg of body weight produces in the majority of experimental rabbits detectable blood levels for periods of 24 to 30 hours. Blood levels ranging from 0.03 to 1.5 units per ml were ob-

‖ In a few instances crystalline procaine penicillin G as well as sodium penicillin G was used as standard. The levels on this basis were at times slightly higher due to the fact that the sensitivity of the test organism (*Streptococcus hemolyticus*) to crystalline procaine penicillin G may be less than its sensitivity to crystalline sodium penicillin G.

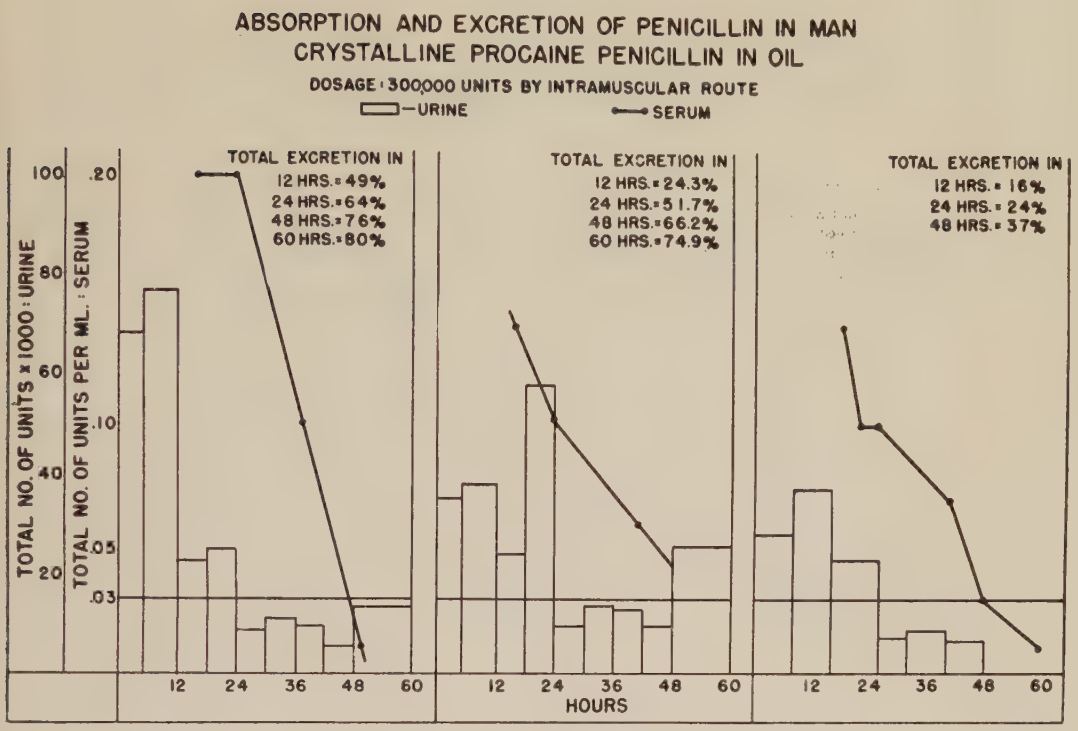

FIG. 3.

served in 80% of animals tested at 18 hours after injection. Sixty-seven per cent showed levels within this range at 24 hours, 50% at 30 hours, 33% at 42 hours, and 25% at 48 hours. Comparable results were obtained with crystalline sodium (potassium, ammonium, or lithium) penicillin G when mixed with procaine hydrochloride in oil in an amount equivalent to that present in crystalline procaine penicillin G. Likewise similar results were observed with other local anesthetics such as procaine buterate and procaine borate. Prolonged but lower levels resulted from the administration of crystalline procaine penicillin dihydro-F in oil, in the same dosage. The use of aqueous rather than oil suspensions of procaine penicillin gave less prolonged penicillin blood levels.

Comparative studies of crystalline potassium penicillin in oil and beeswax, using the same dosage and experimental conditions as those used for procaine penicillin, resulted in detectable blood levels lasting from 18 to 24 hours only.

Preliminary Observations on the Absorption and Excretion of Crystalline Procaine Penicillin in Man. Preliminary observations in man indicate that a single injection of 300,000 units of procaine penicillin G in oil will result in detectable concentrations of penicillin in the blood for periods of 24 to 48 hours, while high concentrations of penicillin may exist in the urine for at least 48 hours (Fig. 3).**

Toxicity of Crystalline Procaine Penicillin. Determination of the toxicity of crystalline procaine penicillins G and dihydro-F in animals is limited by the solubility of the compound. Solutions containing 6,000 units per ml have produced no toxic reaction when administered by the intravenous route to white mice in dosages of 1,400 units per 20 g mouse. Furthermore, rabbits receiving 4,000 units

** Since this paper was submitted for publication, a report has appeared by Herrell and his associates (Herrell, W. E., *et al.*, *Proc. Staff Meet. Mayo Clinic*, 1947, **22**, 567), who have administered procaine penicillin in oil to man in dosages of 300,000 units, and have observed prolonged blood levels similar to those reported herein.

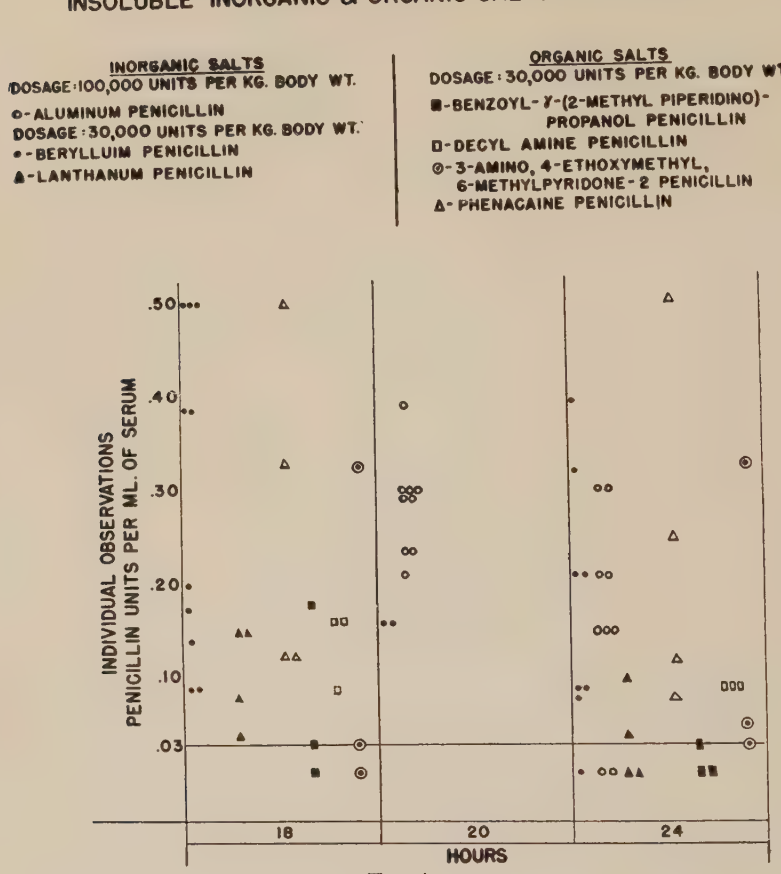

FIG. 4.

intravenously per kg body weight have shown no toxicity. Suspensions containing 40,000 units per ml have caused no reactions in dosages of 40,000 units per 20 g mouse when administered by the subcutaneous route.

It is recognized that the acute mouse toxicity of the soluble salts of penicillins is a direct measure of the concentrations of cation present. In like manner, the minimum lethal dose (LD_0) of crystalline procaine penicillin G (1400 units = 1.35 mg procaine penicillin = 0.56 mg procaine base) is in close agreement with that of procaine hydrochloride (LD_0 = 0.60 mg).

Preliminary observations in man indicate that procaine penicillin in oil is nonirritating on injection and produces no local pain or soreness. It seems probable that crystalline procaine penicillin in oil possesses no greater toxicity than its component parts.

Preliminary Observations on Other Water-Insoluble Salts of Penicillin. Crystalline procaine penicillins G and dihydro-F are water-insoluble salts of penicillin. Their maximum solubilities have been found to be 6,700 units (6.5 mg) and 15,000 units (15.2 mg) per cc, respectively. In view of the fact that procaine penicillin in oil prolongs penicillin blood levels, it seemed likely that other insoluble salts of penicillin might act similarly. That this is true is indicated by the work of Monash[13] who has recently reported detectable blood levels in rabbits at 18 and 24 hours following the intramuscular injection of silver

penicillate, mercury penicillate, and ferric penicillate in oil. Furthermore, Bohls and his associates[14] have indicated that detectable blood levels may be attained for prolonged periods after the intramuscular injection of aluminum-penicillin in oil.

In the present investigation a small series of other water-insoluble organic and inorganic salts of penicillin was tested in experimental rabbits, using the procedure previously described. A dosage of 30,000 units per kg body weight was used throughout. The results are indicated in Fig. 4. Significant levels were observed at 18 and 24 hours in all instances. Beryllium penicillin, the least soluble of the compounds tested, showed the highest and most prolonged levels.

Discussion. Early studies[15–17] on the bacteriostatic and bactericidal properties of the blood of animals treated with aqueous penicillin, as well as subsequent studies on the absorption and excretion of penicillin in man,[18–20] have indicated that penicillin remains in the blood stream for only 2 to 3 hours after injection. These observations have been amply confirmed by subsequent investigators during the past 4 years.

Although it was originally assumed that effective bacteriostatic concentrations of penicillin should be maintained constantly in the circulating blood, as was the case with the sulfonamides, it was soon recognized that 2 injections per day were sufficient for the control of certain experimental infections in animals.[17,21] Furthermore, it was demonstrated by Tillett[22] and more recently by Finland,[23] and by Tompsett and McDermott[24] that pneumococcus pneumonia may be treated successfully with penicillin regimens which afford detectable levels in the blood during only a fraction of each day.

The preponderance of clinical experience with penicillin, however, has been obtained with dosage schedules which maintain continuous or nearly continuous measurable concentrations of penicillin in the blood throughout the period of treatment. For this purpose penicillin in oil and beeswax has been used to advantage.[25–27] It has been amply demonstrated that a single injection of 300,000 units of penicillin in oil and beeswax will maintain detectable concentrations of penicillin in the blood for 12 to 24 hours; however, the reported irritation and hypersensitivity reactions following the administration of this form of penicillin have indicated the need for other methods by which prolongation of penicillin blood levels can be attained.

The fact that such prolongation may result from the use of water-insoluble salts of penicillin, suspended in oil, is of interest. Whereas the majority of these salts are only slowly absorbed, the possible toxicity of many of them limits their use. The toxicity of procaine has been reviewed by Graubard and his associates[28] in a recent communication dealing with the use of procaine intravenously in man. The toxicity of procaine injected intravenously varies with animal species. The minimal lethal dose in rabbits, guinea pigs, and dogs is reported to be in the vicinity of 40 mg per kg body weight; in man, the toxicity of procaine is thought to be dependent upon the

13 Monash, S., *Science*, 1947, **106**, 370.

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21 Dawson, M. H., and Hobby, G. L., *J. A. M. A.*, 1944, **124**, 611.

22 Tillett, W., *et al.*, *Bull. N. Y. Acad. Med.*, 1944, **20**, 142.

23 Finland, M., personal communication, 1947.

24 Tompsett, R., and McDermott, W., personal communication, 1947.

25 Romansky, M., and Rittman, G. E., *Science*, 1944, **100**, 196.

26 Romansky, M., and Rittman, G. E., *New Eng. J. Med.*, 1945, **233**, 577.

27 Romansky, M., Murphy, R. J., and Rittman, G. E., *J. A. M. A.*, 1945, **128**, 404.

28 Graubard, D. J., *et al.*, *N. Y. State J. Med.*, 1947, **47**, 2187.

TABLE II.

Chemotherapeutic Action of Crystalline Procaine Penicillin on Hemolytic Streptococcal (Group A) and Pneumococcal Infections in Mice.

Culture	Total dosage in units	Procaine penicillin in oil: G	Procaine penicillin in oil: Dihydro-F	Procaine penicillin in oil: Mixed	Potassium penicillin G in oil and beeswax	Untreated controls
		Therapeutic effect: % survival				
Streptococcus hemolyticus (C230Mv)	600		75.0			
	450		69.4			
	300	70.0	67.4	90.0	83.3	
	150	51.7	30.0	63.3	43.3	
	50	23.4	20.0	45.0	23.4	8.8
D. pneumoniae (I/230)	500	95.0				
	400	80.0		70.0		
	300	72.5		77.5		
	150	65.0		42.5		
	50	30.0		20.0		

* A minimum of 10 to 15 mice was used for each dilution in each set. One ml of a 10^{-7} dilution contained 1-10 lethal doses of hemolytic streptococci or pneumococci; 10^{-6}, 10-100; 10^{-5}, 100-1,000; 10^{-4}, 1,000-10,000. In all instances mice were injected intraperitoneally with 1 ml of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions of culture.

percentage concentration administered.

The administration of penicillin in 1% procaine hydrochloride has been used frequently to eliminate local irritation and soreness following injection.[29] Crystalline procaine penicillin contains a high concentration of procaine (120 mg per 300,000 units). In preliminary studies it has produced little or no toxicity however.

That crystalline procaine penicillin is a highly efficient antibacterial agent both *in vitro* and *in vivo* has been demonstrated. Furthermore, the fact that this form of penicillin when suspended in oil will prolong blood levels in animals for 24 to 30 hours, or more, following the intramuscular injection of 30,000 units per kg has been shown. Preliminary observations in man indicate that a similar prolongation of blood levels occurs.

The mechanism by which procaine penicillin acts is not known. It is probable that the action is dependent upon at least 3 factors: (1) the low solubility of procaine penicillin in aqueous fluids, (2) the protective action of the oil surrounding the particles of procaine penicillin, and (3) the pharmacological activity of procaine on the tissues at the site of injection.

Conclusions. Crystalline procaine penicillins G and dihydro-F are highly effective antibacterial agents *in vitro* and *in vivo*.

Crystalline procaine penicillins G and dihydro-F, in oil, when injected intramuscularly in rabbits in a single dose of 30,000 units (0.1 cc) per kg body weight, in most instances produces blood levels lasting 24 to 30 hours or longer.

Preliminary observations in man indicate that a single intramuscular injection of 300,000 units of crystalline procaine penicillin G in oil may produce detectable blood levels lasting from 24 to 48 hours while penicillin may be excreted in the urine for at least 48 to 60 hours.

Preliminary observations suggest that the toxicity of crystalline procaine penicillin is probably low.

Other water-insoluble salts of penicillin when suspended in oil also produce marked prolongation of blood levels.

[29] Buckles, D. L., *Bull. U. S. Med. Dept.*, 1947, **7**, 648.

16188

Titration of Small Amounts of Mustard and Other Gases with Bromine and Methyl Red.*

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The quantitative determination of mustard gas and other war gases is difficult since very minute quantities must be analyzed. The method used should also be simple and rapid since it is often necessary to analyze a large number of samples. Mustard gas and the arsenic gases are known to react rapidly with bromine, a reagent which allows extremely accurate titration in very dilute solutions. The usual starch-iodide indicator is not sufficiently accurate for titration with 10^{-4} molar bromine and no satisfactory indicator has been reported.

A number of different oxidizing agents and different indicators have been tried but bromine and methyl red are the most satisfactory in 10^{-4} M solution. Hypochlorite solution may be used in place of bromine. It is much more stable, but reacts with many compounds with which bromine does not.

The method finally developed determines mustard gas, Lewisite, and ethyl or phenyl dichlorarsine within an error of $\pm$ 0.02 ml 10^{-4} molar bromine per ml final volume of the titrated solution. It should be useful for the titration of any compounds which react rapidly with bromine in acid solution.

1. *Description of Samples of Gases Tested.*

Mustard (I)—1 technical, prepared by Levinstein method (Sartori, M., The war gases, New York, D. Van Nostrand Co., Inc., 1940, 223) and redistilled; one C.P. No difference noted.

Ethyldichlorarsine (II)—one C.P. and one technical. Technical titrates slightly lower.

Lewisite (III)—2 technical samples.

Phenyldichlorarsine (IV) — one technical sample.

2. *Preparation of Solutions.* Weighed or measured amounts of I, III, and IV were dissolved in alcohol to give a 10^{-2} M solution. From these, 10^{-4} M solutions were prepared by dilution with water. Solutions of III and I were also prepared in water alone in the same way as those prepared from 10^{-2} M in alcohol.

A 10^{-4} M solution of II was prepared by addition of the gas to water, since II is partially oxidized in alcohol.

These aqueous solutions are referred to as "solutions of the gases," although actually they are solutions of the hydrolysis products of the gases.

3. *Bromine Solution.* Saturated stock solution: 50 ml one M hydrochloric acid is shaken with 10 ml liquid bromine and the solution is kept in a dark glass bottle in the presence of excess bromine. This solution is 0.5 M. The concentration remains constant.

Standardization of bromine solution for titration: When the saturated solution is diluted to 10^{-4} M there is usually a loss of 10-30% in strength due, presumably, to traces of reducing substances in the acid or water used. It is necessary, therefore, to standardize the dilute bromine. This may be done by titration against a solution of 10^{-4} M mustard gas or thiodiglycol in water, or with potassium iodide and 10^{-3} M thiosulfate in the usual way. Ten ml dilute bromine in one N sulfuric acid is added to one ml 10^{-2} M potassium iodide and the liberated iodine titrated with 10^{-3} M thiosulfate, with starch as indicator. One ml 10^{-3} M thiosulfate is equivalent to 0.5 ml 10^{-3} M bromine.

An approximately 10^{-4} M bromine solution was obtained by diluting the saturated bromine solution 1/4,000 with the 0.5 M sulfuric acid used in this work. This dilute bromine solution remains constant within 10% for 24

* This paper is based on work done for the Office of Scientific Research and Development under Contract OEMsr-129 with the Rockefeller Institute for Medical Research.

TABLE I.
Titration of the Various Gases in 0.1 M Sulfuric Acid with Methyl Red and 10^{-4} M Bromine. Initial volume 1 ml.

Gas	Quantity, ml 10^{-4} M	Ml 10^{-4} M bromine Corrected for blank	
—	0	0.05 (blank)	
I	0.1	0.11	
	0.3	0.30	
	1.0	0.95	
	3.0	3.1	
III	0.1	0.11	
	0.3	0.31	
	1.0	1.00	
	3.0	2.95	
		Aqueous sol.—II	10^{-4} M aqueous sol. prepared from 10^{-2} M ETOH sol.
II	0.1	0.09	0.035
	0.3	0.33	0.10
	1.0	0.96	0.40
	3.0	3.10	1.10
IV	0.1	0.09	
	0.3	0.31	
	1.0	1.05	
	3.0	2.95	

hours if kept in dark glass-stoppered bottles. It loses strength rapidly in strong light or if left open. For this reason the burette should be refilled shortly before titrating.

4. *Methyl Red Solution.* Five mg per liter in water.

5. *Concentration Range and Accuracy.* All of the determinations have been carried out with samples of 5 ml or less of a 10^{-4} M solution of the gas titrated with 10^{-4} M bromine, delivered from a 5 ml burette graduated to 0.02 ml. Under these conditions the titrations are accurate to about $\pm$ 0.02 ml of 10^{-4} M bromine per ml final volume of solution, i.e., if the volume at the end of titration is 2 ml the end point is sharp to $\pm$ 0.04 ml. If the final volume is 10 ml the end point is sharp to $\pm$ 0.20 ml. If larger quantities of gas are to be titrated stronger bromine solutions should be used.

Technique of the Determination. A sample of one to 5 ml of the solution is put into a 1.5 x 15 cm test tube and adjusted to approximately 0.1 M sulfuric acid by addition of a few drops of concentrated sulfuric acid. One drop of methyl red is added for each ml of solution. Dilute (10^{-4} M) bromine solution is run in from the burette until the solution becomes nearly colorless. Another drop of methyl red is introduced and bromine added again until the solution is colorless. The end point can be judged best by holding a tube of water alongside the tube of solution which is being titrated. Both tubes should be allowed to rest vertically on white paper and observed from an angle of about 45° to the vertical. The end point is not reversible so care must be taken not to overstep. It is preferable to add the methyl red in two steps owing to partial irreversible oxidation of the indicator by local excess bromine during the titration. Determination of the end point is greatly facilitated by the use of a Fisher fluorescent titration lamp (Fisher Scientific Co., Pittsburgh, Pa.).

The concentration of sulfuric acid or methyl red may vary $\pm$ 50% without affecting the results.

Results. Examples of titrations carried out as described are shown in Table I.

Sources of Error. Any substance which reacts with bromine will evidently interfere with the titration. Hydrogen sulfide and sulfides in general are the most common such substances.

Since rubber tubing and stoppers contain large amounts of sulfides the solutions must not come in contact with them.

Hydrogen Sulfide in Air. Hydrogen sulfide is not absorbed in one M sulfuric acid so that in analyzing samples of air, hydrogen sulfide is not titrated. It may be removed from acid solution by boiling for several minutes. Old sulfide solutions, however, cannot be freed of reducing substances in this way.

Summary. Mustard (I), ethyldichlorarsine (II), Lewisite (III), and phenyldichlorarsine (IV) may be titrated with 10^{-4} M bromine using methyl red as indicator. The method is accurate to about $\pm$ 0.02 ml 10^{-4} M bromine per ml of solution titrated. The reaction of these compounds is that of one mole of bromine per mole of gas.

16189

Carbonic Anhydrase in the Pallium of Primates Compared with that of Lower Mammals.*†

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Carbonic anhydrase reversibly catalyzes the change of carbon dioxide and water to carbonic acid. It occurs in the central nervous system in amounts ranging from a doubtful positive in the cord of the hog[1] to a content in either the cerebellum or cerebrum approximately equal to one-tenth of that found in the blood.[2] None has been found in peripheral nerve of man and dog (unpublished). The content in the spinal cord of mature animals in which it occurs in any considerable amount is well below that of the medulla, pons and higher centers.[3]

Previously reported data indicated a well defined difference in the pattern of the quantitative distribution of carbonic anhydrase in the pallium of the lower animals studied, the dog, the hog and the cat, from that found in man. In the lower animals the cortex held the greater content. In man the pattern was reversed; the white matter immediately below the cortex had a markedly greater enzyme content than the cortex. Exception was found in the motor cortex where more definite approximation to the animal pattern appeared in the leg area.[4]

The present study is an extension of work previously reported. The brains of 4 Rhesus monkeys were examined to see whether the pattern of man is found also in these primates; and additional data on other of the lower mammals, the horse, the steer, the sheep and the rabbit are given. Data on the guinea pig are not included as the subcortical white matter of the brain was too small in amount to be separated successfully from the cortex.

Technique. The technique employed for the determination of carbonic anhydrase was essentially that of Philpot and Philpot[5] as modified by Keilen and Mann.[6] Specific procedures employed[4] and the method used to determine the enzyme activity attributable to the blood in the tissue[7] are given elsewhere.

* This work was aided by a grant from the Supreme Council, Thirty-third Degree, Scottish Rite, Masons and the Northern Jurisdiction, U. S. A.

† I am indebted to Dr. Anna Dean Dulaney of the Pathological Institute of the University of Tennessee for monkey brains.

1 Ashby, W., *J. Biol. Chem.*, 1944, **152**, 235.

2 Ashby, W., *J. Biol. Chem.*, 1943, **151**, 521.

3 Ashby, W., *J. Biol. Chem.*, 1944, **155**, 671.

4 Ashby, W., *J. Biol. Chem.*, 1944, **156**, 323.

5 Philpot, F. J., and Philpot, J. St. L., *Biochem. J.*, 1936, **30**, 2191.

6 Keilin, D., and Mann, T., *Biochem. J.*, 1940, **34**, 1165.

7 Ashby, W., and Chan, D. V., *J. Biol. Chem.*, 1943, **151**, 515.

TABLE I.
Comparison of Carbonic Anhydrase Content of Tissue from Exsanguinated and Non-exsanguinated Guinea Pigs.

	Carbonic anhydrase : Units per gram					
	Control			Exsanguinated		
Tissue	Total	Correction for blood content	Net	Total	Correction for blood content	Net
Cerebrum	(Pig A) 26.7	3.4	*23.3*	(Pig B) 25.0	0.02	*25.0*
Cerebellum	(" C) 40.4	3.0	*37.4*	(" D) 49.3	0.8	*48.5*
Frontal Pole	(" C) 20.0	3.4	*16.6*	(" D) 18.7	0.3	*18.4*

The brain was sampled while fresh—preferably unfrozen. Part of a gyrus was dissected from the pallium and pure white matter beneath the gyrus was taken. This was called "D." The gyrus was then sliced vertically to expose the layer of cortex surrounding the white matter within the gyrus. Approximately the outer half of the cortex was removed with a sharp blade. The remaining inner portion was separated from the white matter by cutting or scraping. These three latter samplings were designated "A," "B" and "C" respectively. (Fig. 1)

Effect of Perfusion. Since the first 3 monkeys received had been used to produce malarial antitoxin and had been bled and perfused when sacrificed until there was little evidence of blood in the brain, it was considered desirable to control the effect of perfusion by using guinea pigs. The degree of anemia produced in these animals was such that the carbonic anhydrase attributable to the blood in the tissue was reduced from 3.0 units or more in the control animals to only 0.8 to 0.02 units in the perfused. The enzyme content of the brain tissue, however, was within the same range in the two series. The results are given in Table I. There was no indication that carbonic anhydrase activity of the brain was lost by perfusion.

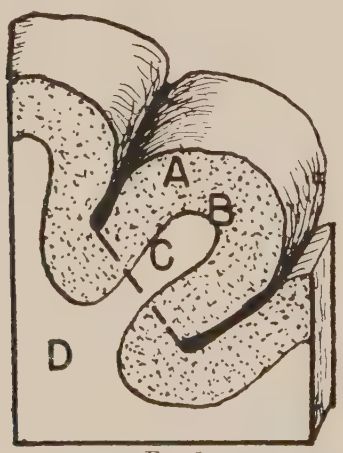

FIG. 1.
Illustrating the method used in dissecting the gyrus into the samples "A," "B," "C," and "D."

Man vs Rhesus Monkey. The carbonic anhydrase content found in the cerebrum of the 4 monkeys studied was low. The content in the cerebellum was 2 to 3 times greater and within the range found in man.

Although the difference between the carbonic anhydrase content of the white and grey matter in the pallium was less in monkeys than in humans, especially in the brains with the smaller amount of enzyme, the pattern of distribution was the one typically found in the human brain. No attempt was made to determine whether, as in the human, the motor cortex of these monkeys gave the reverse distribution typical of the dog, cat and hog. Results obtained by the same technician, from one human and 4 monkey brains are given in Fig. 2 in which the gyrus in the region from which the test material was taken is magnified.

Horse vs Man. Comparisons, in the brain of a horse and of a man, of the carbonic anhydrase content of the cortex with that of the white matter immediately below it, are given in Fig. 3.

In 3 samplings, taken from the parietal area and from the frontal and occipital poles of the brain of the horse, there was definitely less carbonic anhydrase in the white matter

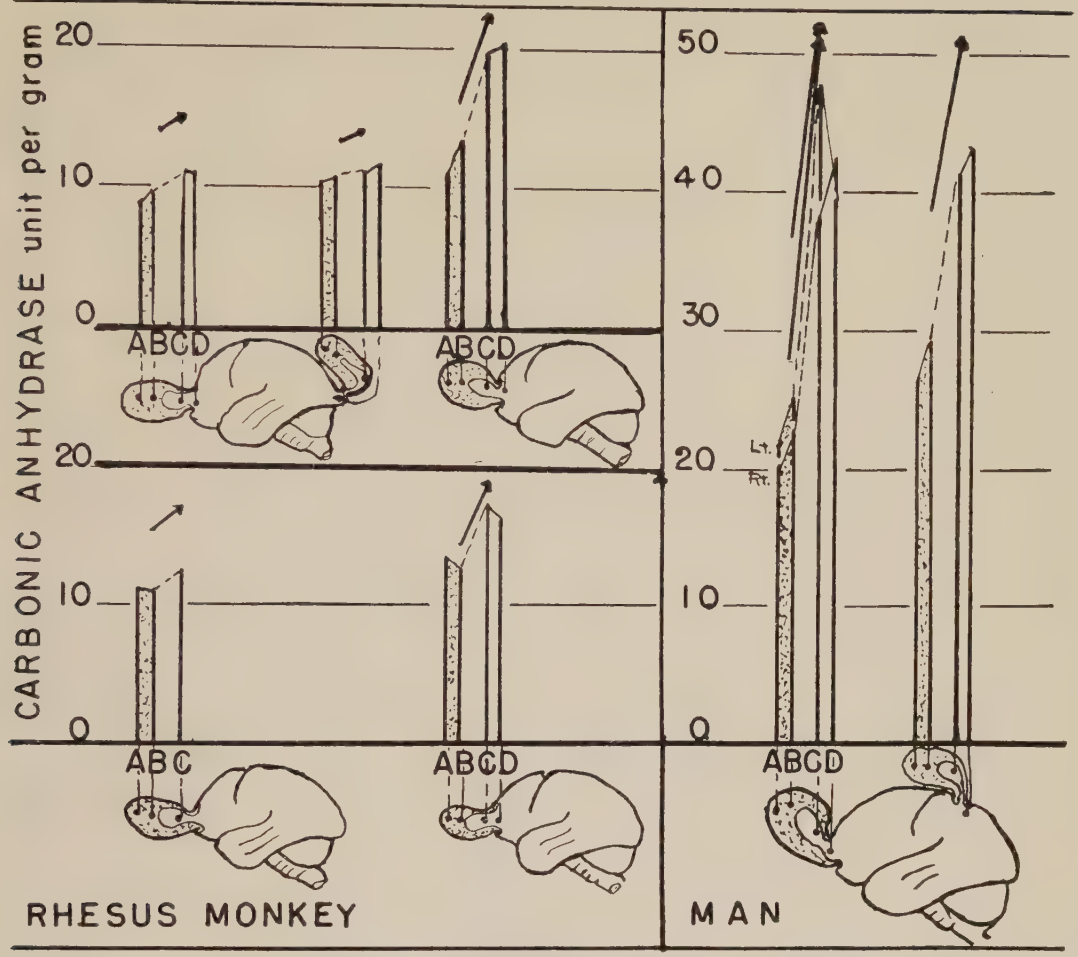

FIG. 2.

Pattern of distribution of carbonic anhydrase between cortex and subcortical white matter, in the cerebrum of a man and Rhesus monkeys.

"A," "B," "C," "D" have the significance indicated in Fig. 1. In the areas from which specimens were taken a gyrus is drawn as though greatly magnified and the "A," "B," "C" samplings are indicated thereon.

below the cortex than in the cortex. In the frontal pole the findings tended to be approximately equal in this brain, in marked contrast with the pattern found in man where, in the frontal pole, there was a great excess of enzyme in the white matter over that of the cortex and in the occipital pole, an approach to equality.[8] The pattern of distribution found in the motor cortex of the human brain was as previously reported, like that found in the horse and in other non-primates studied.

Studies on Sheep, Rabbits and Cattle. Data on the relationship between the carbonic anhydrase content of the cortex and of the white matter below it, in the brains of 2 sheep, 3 rabbits, a calf and a steer, are given in Table II. Sheep I was sacrificed because of its extreme age. Sheep 2 was comparatively young. The rabbit brains were too small to make more than one series of determinations per brain. The calf had been exsanguinated. The steer was an experimental animal and had not been bled. Since its blood was not available, the blood of a cow was used to determine the correction to be made for

[8] Ashby, W., *J. Biol. Chem.*, 1944, **156**, 331.

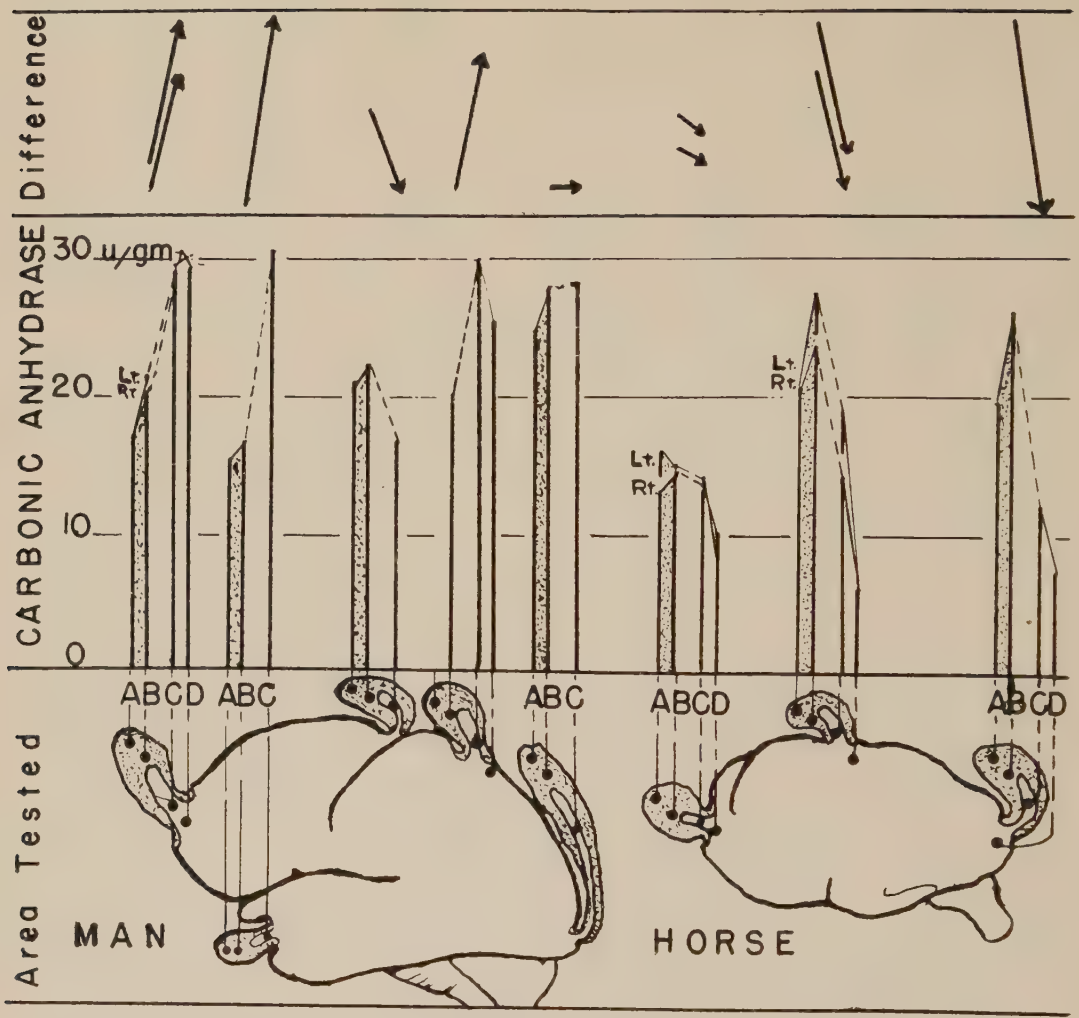

FIG. 3.

Distribution pattern of carbonic anhydrase in the pallium of a man and a horse. In man data on the leg motor area is included. "A," "B," "C," and "D" have the significance indicated in Fig. 1.

blood content. It is probable that the correction in the cortical specimens was too great (Table II).

Summation of Average Results. The data on the relative carbonic anhydrase contents of the cortex and the subcortical white matter are summarized in Table III and compared with findings from 10 frontal and 10 occipital poles from the same human brains. Averaged results from motor areas are also given. Subtraction of the average carbonic anhydrase content of the white matter immediately below the grey matter from that of the grey matter gives a positive number in all species below the primates. In man and Rhesus monkey, on the other hand, a negative quantity is found except in the motor area.

Discussion. It would seem probable that the quantitative pattern of distribution of carbonic anhydrase found in the pallium of man and Rhesus monkey and, so far, not in other animals studied (namely, the dog, hog, cat, horse, cattle, sheep, rabbit) may be part of an evolutionary development peculiar to the primates.

TABLE II.
Comparison of Carbonic Anhydrase Content of Cortex with That of Sub-cortical White Matter in Sheep, Rabbit, and Cattle.

Animal	Area	Carbonic anhydrase, units per gram, corrected for blood content: correction in parentheses			
		Cortex		Sub-cortical white	
		A	B	C	D
Sheep 1.	Frontal	17.1 (1.1)		16.3 (0.7)	
	Occipital	18.2 (0.9)		12.2 (0.7)	
" 2.	Frontal	38.2 (0.9)		19.0 (1.4)	15.8 (0.7)
	Precentral	34.0 (4.5)		18.0 (2.4)	
	Postcentral	39.0 (2.7)		16.6 (1.4)	
	Occipital	39.0 (4.5)		25.2 (1.8)	14.7 (1.4)
Rabbit 1.		16.8 (1.3)		13.5 (1.8)	
" 2.		35.1 (2.0)	24.1 (1.5)	22.3 (0.5)	
" 3.		37.9 (5.6)	32.9 (5.6)	24.7 (3.5)	
Calf		12.5 (0.2)	12.4 (0.1)	8.5 (0.1)	7.0 (0.1)
"		11.1 (0.1)	11.4 (0.1)	8.1 (0.1)	
Steer		12.7 (8.0)	12.9 (6.5)	11.8 (5.0)	12.5 (1.4)
"		18.2 (5.1)	16.0 (4.9)	12.9 (4.0)	12.0 (1.6)
"		11.6 (6.7)	9.4 (5.0)	9.9 (1.6)	8.7 (1.3)

TABLE III.
Comparison of Carbonic Anhydrase Contents of the Cortex and the Sub-cortical White Matter in Primates and Lower Animals.

Animal	No. of comparisons	No. of animals	Average findings Units per gram		Difference %
			Cortex	White matter	
Rabbit	3	3	27.3	20.2	26
Steer	5	2	12.8	10.2	20
Sheep	4	2	30.9	18.0	42
Horse	6	1	20.5	14.9	27
Cat	6	4	39.2	24.9	36
Dog	6	2	33.7	14.4	57
Hog	9	5	34.8	26.4	24
Rhesus monkey	5	4	11.2	14.9	—33
Man					
Frontal pole	10		19.2	29.7	—55
Occipital pole	10		25.3	29.2	—15
Motor cortex	11		33.1	26.2	21

A tendency toward an inverse relationship among species between the size of the animal and the rate of metabolism of its brain has been reported.[9] In the series studied, the brain of the horse, which most nearly approaches that of man in size, shows a cortical content of carbonic anhydrase somewhat below that in the human brain. Therefore the larger amount of enzyme found in the subcortical white matter of man as compared with that found in the cortex might be regarded as an increase of the enzyme in the white matter beneath the cortex, rather than as a decrease within the cortex.

In the occipital pole in man, where there is a point to point projection from the geniculate nucleus, there is a decreased difference between the enzyme content of the cortex and the white matter beneath it. In the motor cortex, where the white matter consists largely of efferent fibers, the usual pattern of distribution peculiar to man and the Rhesus monkey is not present. Here, especially in the leg area, the white matter below the cortex

9 Page, I. H., *Chemistry of the Brain*, Springfield, Ill., Thomas, 1937.

contains definitely less enzyme than the cortex and the pattern of distribution is like that in the lower animals.

In this region below the cortex, which in the primates shows the increased enzyme content, are found fibers which make extrinsic cortico-cortical connections. The coincidence of this pattern of distribution with the development in the primates of the capacity for wider mental association, as found in man, may be of significance. If this difference in the pattern of distribution proves to be tenable after further study, it would differentiate qualitatively between the brain of primates and that of the lower animals.

Summary. Within the pallium a quantitative distribution pattern of carbonic anhydrase, not found in the dog, cat, hog, horse, sheep, cattle and rabbit, has been found in man and the Rhesus monkey. Its possible significance is discussed.

16190

Purification of the Resin Amberlite IR-100 for Blood Coagulation Studies.*

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The phenol-formaldehyde resin, Amberlite IR-100, which can remove calcium from blood completely by virtue of its ion exchange property[1] has become a valuable agent for quantitative studies of calcium in the coagulation mechanism.[2,3] Since certain lots of Amberlite have been found to contain impurities which interfere with coagulation, an improved method of purification had to be devised. Several studies have been carried out with the purified product to determine further whether Amberlite induces any change in blood other than the removal of calcium.

Experimental. 1. *Purification of Amberlite.*‡ Twenty grams of Amberlite were covered with 100 cc of 5% (by volume) sulfuric acid and the mixture boiled and stirred vigorously for 5 minutes. The resin was washed with distilled water by decantation until the wash water was no longer acid. The material was then treated with 100 cc of 5% sodium carbonate solution, heated to approximately 70°C and thoroughly stirred. The deeply colored supernatant fluid was poured off and the process repeated until little coloring matter could be extracted. The resin was finally washed with warm distilled water until all traces of sodium carbonate were removed.

The cation-exchange resin was prepared for reaction by adding to it 250 cc of 5% solution of sodium chloride, stirring vigorously for 30 minutes and allowing the mixture to stand for 60 minutes more. The resin was then washed with distilled water and filtered by suction until the wash water no longer contained chloride ions. The solid was dried at 37°C.

2. *Decalcification with Amberlite.* To prevent incipient coagulation, blood obtained by venipuncture with minimal trauma was drawn into a syringe coated with Silicone and immediately passed through a column of 3 g of Amberlite, an amount sufficient for complete decalcification of 10 cc of blood. The

* This work was supported by a grant from the United States Public Health Service.

† Department of Internal Medicine, University of Roma; at present, Senior Research Fellow, National Institute of Health.

1 Steinberg, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 124.

2 Quick, A. J., *Am. J. Physiol.*, 1947, **148**, 211.

3 Stefanini, M., and Quick, A. J., *Am. J. Physiol.*, in press.

‡ Mr. James C. Winters of the Resinous Products and Chemical Company offered valuable suggestions.

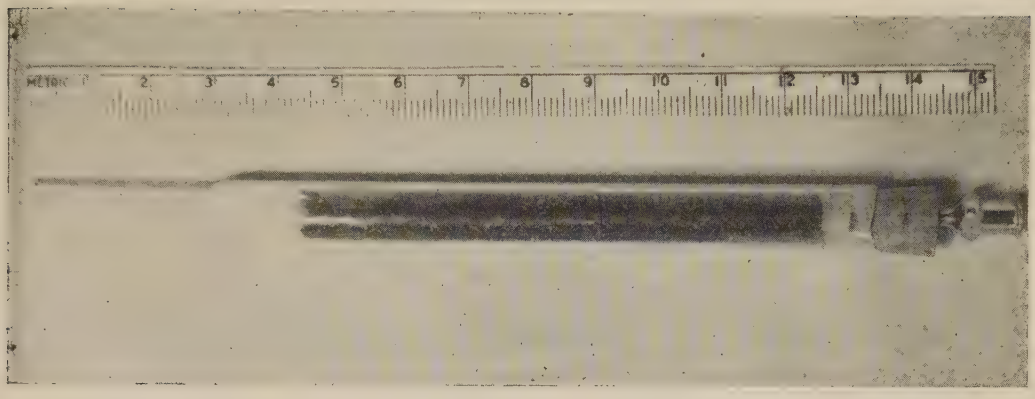

FIG. 1.
Tube Employed for Decalcifying Blood with Amberlite. The tube, the plug of glass wool, and the needle (No. 16) were all coated with Silicone; the cork with collodion.

tube used was approximately 1 x 15 cm and was coated with Silicone§ (Fig. 1). The decalcified blood was collected in a test tube coated with Silicone, then passed twice more through the same Amberlite. This resin can be re-used several times if it is washed free of all traces of blood with distilled water immediately after each use, then treated with 5% sodium carbonate and recharged with sodium chloride.[1]

Properties of the blood decalcified with Amberlite. Steinberg's original findings[1] that the blood was little altered, except by removal of calcium, were confirmed. If any calcium remained, the amount was too small to be determined by ordinary analytical methods. Since dog blood thus decalcified failed to clot after the addition of thromboplastin, the calcium remaining must have been considerably less than 0.0001 M, a concentration still adequate to produce rapid clotting.[2] The thromboplastin used in this experiment and in all of our determinations of prothrombin time of plasma decalcified with Amberlite was made calcium-free. This was done by careful trituration of the rabbit brain from which it was prepared, with 0.02 cc of a 0.1 M solution of sodium oxalate per g of fresh weight, before dehydration with acetone.

Curiously, the sedimentation rates determined with blood decalcified with Amberlite were consistently lower than with citrated or heparinized blood (Table I), thus confirming the findings of Steinberg. This observation may ultimately help to explain the sedimentation behavior of blood.

The clotting time of whole blood decalcified with Amberlite and recalcified with an optimum concentration of $CaCl_2$ (0.00386 M) appears to be much shorter than that of the original blood. For example, the clotting time of 8 minutes 15 seconds for a sample of native human blood was reduced by treatment with Amberlite and recalcification to 3 minutes and 30 seconds. However, a similar result is obtained with blood recalcified after oxalation or citration.

As shown by Steinberg, little or no modification of the prothrombin activity, as measured with the one stage method, takes place in blood decalcified with Amberlite; the fibrinogen content is slightly decreased (10-15%) but not sufficiently to cause any change in the coagulability. Human plasma that has been decalcified with Amberlite after the removal of prothrombin and fibrinogen can restore to normal the apparently delayed prothrombin time of stored human oxalated plasma.

The antithrombin activity of Amberlite plasma was compared with that of citrated or oxalated plasma by two methods. In the first, thrombin of decreasing strength was added to a constant amount of oxalated or Amberlite plasma according to the method

§ Methyl-chloro-silane; Dri Film No. 9987 (General Electric).

TABLE I.

Sedimentation Rate of Citrated, Heparinized, and Amberlite Human and Dog Blood After 1 and 24 Hours.

Westergren's technic was used. Values given are in mm.

Subject	Human blood				Dog blood			
	1	2	3	4	1	2	3	4
After 1 hr								
Citrated blood	10	14.5	23	17.5	2	2	2.5	3
Heparinized "	9.7	15	24	18	2	2	3	3
Amberlite "	4	9	15	10.5	1	1	1	2
After 24 hr								
Citrated blood	57	73	99	91	11	15	22	24.5
Heparinized "	58	75	102	94	10	16	20.5	26
Amberlite "	49	64	71	62	6	10	13	14.5

TABLE II.

Antithrombin Activity of Oxalated and Amberlite Plasmas of Man, Dog, and Rabbit. Each figure in the table represents the average clotting time in seconds obtained in several experiments.*

Dilution of thrombin		Full strength	½	¼	⅛	1/16	1/32
Human plasma	Amberlite	3	4	6	9	15.5	27
	Oxalated	3	4	6.5	9.5	17	29
Dog "	Amberlite	3	4	6.5	12	17.5	32
	Oxalated	3	4	7	11.5	19.5	35
Rabbit "	Amberlite	3.5	4.5	7	12.5	20.5	38
	Oxalated	3.5	5	7.5	13	22	39

* 0.1 cc of thrombin was added to 0.2 cc of the plasma. The tubes were incubated in a water bath at 37°C. The thrombin was always prepared from human oxalated plasma and stored for an hour or longer, until its activity became constant. It was diluted as required with distilled water.

of Quick.[4] In the second, an equal volume of Quick's "full strength" human thrombin[5] was incubated with Amberlite, oxalated or citrated plasma and the rate of decrease in thrombin activity was measured using normal oxalated plasma as a source of fibrinogen. Details are appended to the tabulated data (in Tables II and III).

The results recorded in Table II show plainly that the clotting response of Amberlite plasma to varying concentrations of thrombin is the same as that of oxalated plasma. Therefore, the Amberlite does not introduce or reduce any agent antagonistic to thrombin, nor does it affect the stability of fibrinogen. When thrombin is incubated with Amberlite plasma (Table III), its inactivation is less pronounced than when it is incubated with oxalated plasma, but essentially the same as with citrated plasma. Further investigation is required to provide an explanation.

Summary. 1. A method of purification of resin Amberlite IR-100 was devised. When blood was treated with the purified resin a decreased sedimentation rate was found, with morphological, chemical, and physical properties otherwise practically unmodified.

2. Of the factors involved in blood coagulation, prothrombin, fibrinogen and the labile factor described by Quick[6] apparently were not appreciably altered by the decalcification with Amberlite. With optimal recalcification, the clotting time of the treated blood was much shorter than that of native blood, but this is also true for oxalated and citrated blood.

3. A reduced antithrombin activity was observed in Amberlite plasma upon incubation with "full strength" thrombin. Since

[4] Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Charles C. Thomas, Springfield, Ill., 1942, p. 319, 321.

[5] Quick, A. J., *Am. J. Physiol.*, 1936, **115**, 317.

[6] Quick, A. J., *Lancet*, 1947, **2**, 379.

TABLE III.

Effect of Incubation with Citrated, Oxalated, and Amberlite Plasmas of Man, Dog, and Rabbit on Activity of Full Strength Human Thrombin.

Each figure in the table represents the average clotting time in seconds obtained in several experiments.*

Length of incubation (sec.)		60	120	180	240	300
Human plasma	Amberlite	8	11	13.5	29.5	34
	Citrated	12	14	16	21	29
	Oxalated	12	23	64	450	†
Dog "	Amberlite	8.5	12	18.5	27	52
	Citrated	11	13	17.5	25	34
	Oxalated	13	26.5	49	90	225
Rabbit "	Amberlite	9	11.5	14	22	40
	Citrated	11	12.5	15	20.5	34
	Oxalated	11	35	49	58.5	87

* Equal volumes of full strength human thrombin and of the plasma to be tested were incubated in a water bath at 37°C; the clot was wrapped about a glass rod coated with collodion and removed. After the incubation, 0.1 cc of the mixture were added to 0.2 cc of oxalated homologous plasma as a source of fibrinogen and the clotting time was determined and recorded.

† No clotting in 1 hr.

citrated plasma responded similarly, the behaviour may be linked with the mechanism of decalcification of the 3 agents.

16191 P

Effect of Polyoxyalkylene Sorbitan Monooleate on Blood Cholesterol and Atherosclerosis in Cholesterol-Fed Rabbits.*

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In an attempt to alter the solubility or colloidal stability of cholesterol *in vivo* and thus modify the development of experimental cholesterol atherosclerosis, Hueper[1] administered several commercial detergents to cholesterol-fed rabbits and observed no striking alteration in blood cholesterol levels or degree of atherosclerosis.

This report deals with the effect of oral polyoxyalkylene sorbitan monooleate, Tween 80,‡, a surface-active agent of low toxicity, on the level of blood cholesterol and on the development of atherosclerosis in cholesterol-fed rabbits.

Experimental. Adult male and female rabbits averaging 3.2 kg in weight were fed a stock diet of Rockland rabbit pellets to which were added either:

A. Cholesterol—1 g; peanut oil—3 cc; Tween 80—10 cc.

B. Cholesterol—1 g; peanut oil—3 cc.

C. Tween 80—10 cc.

The ingredients were added separately to the stock diet in individual containers, and thoroughly mixed.

In Experiment 1, 6 rabbits were fed Diet A, and 5 Diet B daily. In Experiment 2, 12

* Aided by a grant from the United States Public Health Service.

† This work was done during the tenure of a Life Insurance Medical Research Fellowship.

1 Hueper, W. C., *Arch. Path.*, 1944, **38**, 381.

‡ Manufactured by the Atlas Powder Co., Wilmington, Del.

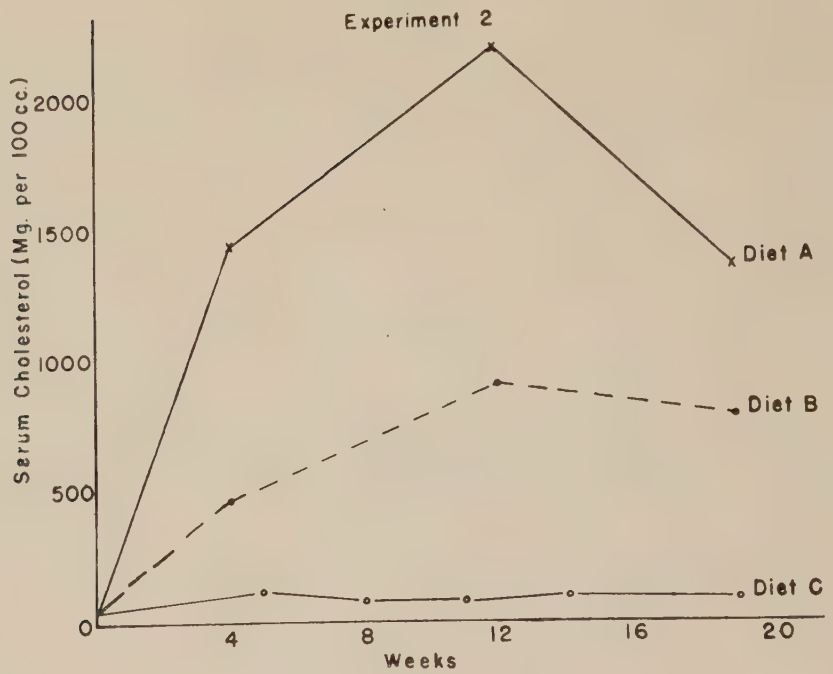

FIG. 1.
Mean Serum Cholesterol Levels.
Diet A—Cholesterol, peanut oil, and Tween 80 (12 rabbits).
Diet B—Cholesterol and peanut oil (11 rabbits).
Diet C—Tween 80 (3 rabbits).

rabbits were fed Diet A, and 11 Diet B 3 times a week, and 3 additional rabbits were fed Diet C daily. Blood was drawn from the marginal ear vein, and serum total cholesterol determined by a modification of the method of Bloor, Pelkan, and Allen.[2] Tween 80 *per se* does not influence the colorimetric determination of cholesterol, as it does not give the Leibermann-Burchard reaction itself or when added to serum *in vitro*. The degree of atherosclerosis of the aorta was determined by gross examination of the fresh specimen, and after fixation and staining of the entire aorta in Sudan IV.

Results. In Experiment 1, at 4, 7, and 10 weeks the mean serum total cholesterol levels of rabbits fed Diet A (cholesterol, peanut oil, and Tween 80) were 1,490, 2,725, and 2,150 mg per 100 cc respectively, as compared with 725, 975, and 1,160 mg per 100 cc for the rabbits fed Diet B (cholesterol and peanut oil). Fig. 1 summarizes the results of Experiment 2. The serum total cholesterol levels reached a peak at 12 weeks with a mean value of 2,215 mg per 100 cc (range 900-4,350) for rabbits on Diet A, and 880 mg per 100 cc (range 285-2,205) for rabbits on Diet B. The difference between the two groups is more than 6 times the probable error, and is statistically significant. Rabbits fed Diet C (Tween 80) showed at first a very slight rise in blood cholesterol, which then returned to and remained within the normal range. The blood cholesterol levels of the rabbits fed Diet B were in approximately the same range as those previously reported in cholesterol feeding experiments (Weinhouse and Hirsch;[3] Dubach and Hill[4]).

The action of Tween 80 in augmenting the blood cholesterol level is probably due, in

[2] Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, 1922, **52**, 191.

[3] Weinhouse, S., and Hirsch, E. F., *Arch. Path.*, 1940, **30**, 856.

[4] Dubach, R., and Hill, R. M., *J. Biol. Chem.*, 1946, **165**, 521.

part at least, to better emulsification of cholesterol in the intestinal tract and more efficient absorption. Studies now in progress using parenteral administration of Tween 80 indicate that because of its detergent properties it may also have a more direct influence on the level of cholesterol and other lipids in the blood.

Rabbits fed cholesterol plus Tween 80 developed atherosclerosis of the aorta more uniformly, at an earlier date, and of a slightly more severe degree than the cholesterol-fed controls. Animals fed only Tween 80 had no atherosclerosis.

Studies are now in progress to determine whether Tween 80 can effect resorption of experimental atherosclerosis.

Summary. Rabbits fed Tween 80 and cholesterol developed blood cholesterol levels that were 2 to 3 times as high as those obtained by cholesterol feeding alone, and also exhibited an earlier and somewhat more severe degree of atherosclerosis.

16192

V. Long-term Maintenance of Two Strains on Synthetic and on Stock Diets.*

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Nielsen and Black[1] have reported that mice require a dietary source of biotin and folic acid during their early period of growth, and that this requirement is increased by incorporating sulfasuxidine in the ration. We[2] have observed that mice of 2 highly inbred strains grow well on synthetic rations containing no added biotin and folic acid. We have also shown, however, that one or both of these factors may be essential for satisfactory reproduction and lactation.[3] Unpublished work in this laboratory has revealed that mice of the C_{57} strain may be maintained on a synthetic diet with 0.75% sulfasuxidine without added biotin and folic acid but with p-aminobenzoic acid for about 6 months without showing outward changes other than slight alopecia and achromotrichia. The discrepancy between our results and those of Nielsen and Black and the fact that the literature contains few reports of long-term maintenance of mice on highly purified diets led us to analyze the records of our mouse colony in which animals have been maintained on various stock diets and synthetic rations.

Methods. Two strains of mice (the A strain, with high incidence of spontaneous mammary tumors, and the C_{57} with low tumor incidence) have been employed in these studies. At weaning the animals were placed in individual screen-bottom cages and their growth rates measured until they were 10 weeks old. Thereafter they were used for reproduction and lactation studies as previously reported.[3] During the rest intervals between matings or after the end of the reproductive studies 2 or 3 animals were housed together. The stock rations employed were Purina Dog Chow and later Purina Laboratory Chow. The synthetic diet (No. 101)[3] contained dextrose 60%, "vitamin-free" casein 23%, hydrogenated cottonseed

* This investigation was supported by grants from the Nutrition Foundation, Inc., the Anna Fuller Fund, and the American Cancer Society, on the recommendation of the Committee on Growth of the National Research Council.

1 Nielsen, E., and Black, A., *J. Nutrition*, 1944, **28**, 203.

2 Fenton, P. F., Cowgill, G. R., and Stone, M. A., *Yale J. Biol. and Med.*, 1947, in press.

3 Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, 1947, **33**, 703.

TABLE I.
Summary of Observations.

Strain	C_{57}				A			
Sex	Male		Female		Male		Female	
Diet	Stock	101	Stock	101	Stock	101	Stock	101
No. of animals	14	8	30	11	9	19	21	28
Achromotrichia (slight)	2	1						
Alopecia (slight)		4	3	2	1	2		2
Skin ulceration						2		2
Eye involvement						6		1
Moist dermatitis				2		5		4
Massive fecal crusts						4		2
No. of animals with tumors							14	16
Avg time of onset—months							11½	10
Avg No. of litters born			4.3	2.7			4.0	1.9
No. of deaths								
Fecal crusts						4		2
"Tumors"							4	5
Incisor overgrowth				1				
Avg wt when sacrificed—g	28.2	30.3	29.0	26.8	27.8	25.2	—	—

oil 10%, salts 5%, roughage 2%, 8 vitamins of the B complex (thiamine, niacin, pyridoxine, riboflavin, pantothenate, choline, inositol, and p-aminobenzoic acid), cod liver oil concentrate, α-tocopherol, and linoleic acid. The animals on synthetic rations were given various supplements during the reproduction study;[3] these, however, were only administered for relatively brief time periods. Because of lack of space, it was necessary to sacrifice most of the animals when they were about a year old.

Results. The pertinent observations made on these animals in the course of their existence or at the end of the year period are summarized in Table I. Mice of the A strain maintained on synthetic diet showed numerous symptoms which they did not develop on the stock ration. Most of these changes were not observed in the C_{57} strain maintained on either diet. The eye lesions consisted of inflammation and denudation of the surrounding skin. Occasionally a dried exudate was observed in this area. Animals showing these conditions were usually hesitant to open their eyes when disturbed. The moist dermatitis which was observed almost exclusively in the A strain on synthetic diet was usually found below the chin and extended caudad for a distance of several centimeters. No hair was seen in the involved areas. Skin ulcerations, when present, were usually on the lateral body areas and on the legs. The formation of massive fecal crusts over the anus was observed in 6 animals of the A strain on synthetic diet. This condition, which has always been fatal, has also been observed in the offspring of the generation of mice reported here. We have seen this happen only in A strain mice on synthetic diets. It has even occurred in the animals subsisting on the most complete synthetic diets which we have studied.

The causes of deaths in each group of animals are arranged in the table in 4 classes. When the cause of death is described as "tumor," we mean only that the animals in question had a sizable mammary tumor which could have been the cause of death. No extensive autopsies were performed on the dead animals.

Discussion. We have not found under the conditions of our experiments that the mouse requires a dietary source of biotin or folic acid for early growth; nor have we observed any deficiency symptoms during the early period of life as did Nielsen and Black.[1] A possible explanation of this discrepancy may lie in the fact that Nielsen and Black carried out special alcohol extractions of their casein and sucrose, while we used commercial "vitamin-free" casein and dextrose C.P. without further treatment. Biotin assays on our diet 101 showed it to contain less than one μg of biotin per 100 g of ration. The fact that

Nielsen and Black did not incorporate p-aminobenzoic acid in their rations does not offer a convincing reason for the discrepancy. It seems much more likely that the difference is due to the nature of the dietary carbohydrate. We have used dextrose while Nielsen and Black used sucrose. This aspect of the problem is now being further investigated. Another possible explanation at the moment seems to be one of strain difference. Our evidence[4,5] very strongly supports the view that there is a clear strain difference in the nutritional requirements of the C_{57} and the A strain. The mice used by Nielsen and Black may have possessed such a high requirement for biotin and folic acid that they showed deficiency symptoms during a very early period of life.

The difference in the time required for A strain females to develop mammary tumors on the stock and the synthetic diets is highly suggestive and is being investigated more thoroughly.

The much greater incidence of symptoms and deaths of A strain mice on synthetic diets suggests again their greater nutritional requirements. We have shown in this laboratory[4,5] that this strain requires more riboflavin and pantothenic acid for growth than does the C_{57} strain. The findings reported here suggest also a greater need for biotin or folic acid or both in order to maintain the animal in good health during later life. The absence of possible unknown nutritional factors cannot be altogether overlooked. We have found[6] that mice of the C_{57} strain on synthetic diets had greater cecal contents and a greater bacterial population than did mice of the A strain. This strain difference was not observed in animals on stock ration.

Summary. Two strains of highly inbred mice, one tumor-susceptible and the other tumor-resistant, were maintained for a period of about one year on a stock diet or on a synthetic ration adequate for good growth performance. The tumor-susceptible A strain on synthetic diet showed numerous deficiency symptoms not seen on stock rations and not shown to any great extent by C_{57} mice on either stock or synthetic diets.

4 Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, 1947, **34**, 273.

5 Fenton, P. F., and Cowgill, G. R., *Fed. Proc.*, 1947, **6**, 407.

6 Gall, L. S., Fenton, P. F., and Cowgill, G. R., in press.

16193 P

Antagonism of Sulfadiazine Inhibition of Psittacosis Virus by p-Aminobenzoic and Pteroylglutamic Acids.*

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Studies on the chemotherapeutic action of the sulfonamides on certain bacteria[1] have shown that p-aminobenzoic acid (PABA) and pteroylglutamic acid (PGA) antagonize the inhibition of growth of these organisms by sulfonamide drugs. The demonstration of sulfadiazine (SD) inhibition of the growth of certain strains of psittacosis virus[2] suggests the likelihood that PABA and PGA might be active antagonists for this inhibitory effect of the sulfonamides.

A yolk sac passage strain of psittacosis

* Aided by a grant from the National Foundation for Infantile Paralysis.

† Senior Fellow in the Medical Sciences of the National Research Council.

1 Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, 1947, **170**, 133.

2 Early, R. L., and Morgan, H. R., *J. Immunol.*, 1946, **53**, 151.

virus (6BC) was employed using the same experimental techniques previously described[2] for the determination of the effect of SD on the growth of the virus. PABA and PGA were prepared as sterile solutions of their sodium salts in distilled water. These solutions were mixed with the proper amounts of a sterile solution of sodium sulfadiazine just before injection in 0.25 ml amounts into the yolk sac of 7-day-old embryonated eggs. This was followed in ½ hour by the injection of a yolk sac suspension of psittacosis virus diluted to contain 10,000 LD_{50} in 0.25 ml. The doses of the various compounds are recorded in weights of PABA, PGA and SD.

Preliminary experiments have indicated that the minimal amounts of the two compounds required to antagonize the inhibitory effect of 2.5 mg SD on the growth of the virus was 0.005 mg PABA and 0.05 mg PGA. Following the establishment of these relationships, a series of experiments was carried out to determine the amounts of the 2 inhibitors required to antagonize the action of larger amounts of SD. Representative results are presented in Table I, using amounts of PABA and PGA twice and ten times the minimal doses noted above.

There is a direct relationship between the amount of SD used and the amount of PABA required to antagonize its inhibitory action. This suggests a competitive inhibition of the action of SD. In contrast, a given dose of PGA is demonstrated to be actively antagonistic for increasingly larger doses of SD which suggests a noncompetitive antagonism. These data suggest that, as is the case with certain bacteria which synthesize PGA,[1] the primary action of SD on psittacosis virus is directed against the incorporation of PABA into PGA by the virus because of the direct relationship between the amount of PABA required to antagonize a given amount of SD. When PGA is supplied, even large doses of SD fail to inhibit the growth of the virus. These findings suggest that psittacosis virus (6BC strain) synthesizes PGA and that this vitamin is required for its growth. Studies are now underway to elucidate this possible metabolic activity of the virus.

TABLE I.
Antagonism of Sulfadiazine Inhibition of Psittacosis Virus by *p*-Aminobenzoic Acid and Pteroylglutamic Acid.

SD, mg	Inhibitor agent, mg		No. eggs	% survived 10 days*
0.5	PABA	.01	7	0
2		,,	8	0
5		,,	6	0
10		,,	19	47
25		,,	13	85
50		,,	6	100
0.5	PABA	.05	5	0
2		,,	8	0
5		,,	8	0
10		,,	18	0
25		,,	11	8
50		,,	4	100
0.5	PGA	.1	7	0
2		,,	8	0
5		,,	6	0
10		,,	16	0
25		,,	11	0
50		,,	8	0
0.5	PGA	.5	8	0
2		,,	7	0
5		,,	7	0
10		,,	20	0
25		,,	10	0
50		,,	7	0
0.5		—	16	82
5		—	24	96
—	PABA	5.0	8	0
—	PGA	10.0	10	0
—		—	16	0
		Drug Controls.†		
50		—	8	85
—	PABA	5	20	95
—	PGA	10	19	95

* 10,000 LD_{50} injected into each egg.
† Not infected.

Summary. The chemotherapeutic action of sulfadiazine on psittacosis virus (strain 6BC) is antagonized competitively by p-aminobenzoic acid and noncompetitively by pteroylglutamic acid. The implications of these findings with regard to the metabolic activities of this virus are discussed.

16194 P

Microbiological Determination of Apparent Free Methionine in the Blood of Children.

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Few studies on the blood levels of methionine, one of the amino acids indispensable for man, have been reported. By the use of the microbiological method of assay, Harper, Kinsell, and Barton,[1] found that the mean fasting level of apparent free *L*-methionine in the plasma of 11 normal male adults, was 0.85 ± 0.09† mg per 100 ml with a range from 0.46 to 1.48. However, there has been no published information concerning the values in the newborn or older child. The purpose of this communication is to present the results of studies on the blood of (1) hospitalized "normal" children; (2) hospitalized children with various diseases; and (3) on the blood of the umbilical cord at the time of delivery.

Method. All of the children were in a postabsorptive state. Blood samples from them were obtained from either the antecubital or jugular vein. The "normals" had been hospitalized for the correction of non-infectious orthopedic difficulties. In the case of the newborn infants, cord blood was taken from the maternal end of the umbilical cord as soon as possible after delivery. Heparin was used as the anticoagulant in each case.

Proteins were removed from the plasma by heating and adding 5% acetic acid.[1] *L*-methionine in the filtrates was determined microbiologically, with *Leuconostoc mesenteroides P-60* as the assay organism.[2] One ml of the basal medium recommended by Harper *et al.*[1] was used in each determination; the plasma filtrates were added at 3 different levels (0.4, 0.6, and 1.0 ml); and the final volume was adjusted to 2 ml. All tubes were incubated for 72 hours and acid production was determined by electrometric titration with NaOH.

Results. The mean concentration of apparent free *L*-methionine in the plasma of "normal" children was 0.29 ± 0.02 mg per 100 ml of plasma. The range was from 0.19 to 0.47. The results are plotted in Fig. 1A. The difference between these levels and those found by Harper *et al.* in normal adult males with the same method of assay is statistically significant. Thirty-three hospitalized children, who were suffering from various diseases, had plasma methionine levels which were in the same range as those of the "normal" children. The mean was 0.42 ± 0.05 mg per 100 ml of plasma with a high of 1.07 and a low of 0.08 (Fig. 1B). At the time of testing, many of the children were convalescing, but there was no significant difference between their levels and those of the acutely ill ones.

Of 30 umbilical cord bloods tested, the mean level was 0.86 ± 0.04 mg per 100 ml of plasma (Fig. 1C). These results were in the same range as those found in adult males and were more than 3 times as high as those in "normal" children or those who had been ill.

Discussion. The foregoing data indicate that the plasma levels of apparent free *L*-methionine in the blood in the umbilical cord at birth is approximately the same as the fasting level reported for adult males.

At least two factors may operate to account for the lower plasma levels found in the children: (1) The main need for protein in the adult is to repair body tissue. The growing child needs an additional supply for growth and for the formation of new tissue. The increased demands in the child may lead to a greater rate of disappearance of essential

* With the technical assistance of Mrs. Tilly B. Leake.

1 Harper, H. A., Kinsell, L. W., and Barton, H. C., *Science*, 1947, **106**, 319.

† Throughout this paper the figures following the symbol "±" refer to the standard error of the mean.

2 Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *J. Biol. Chem.*, 1946, **163**, 577.

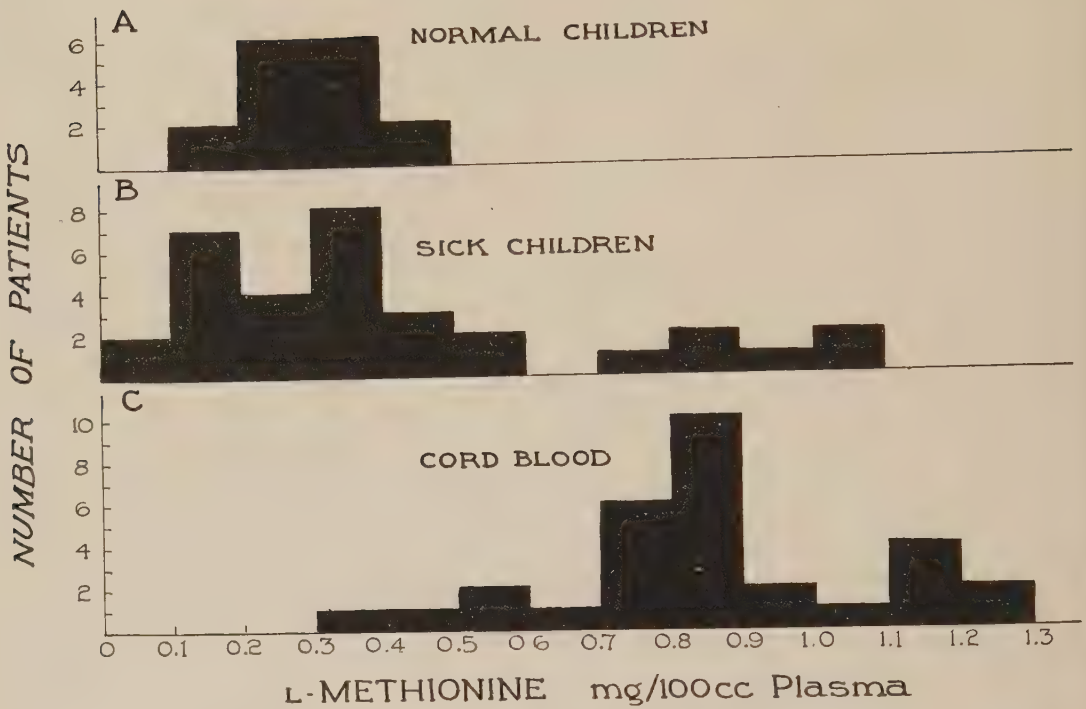

FIG. 1.

Apparent free *L*-methionine in plasma: (A) in hospitalized "normal" children; (B) in hospitalized children with various diseases; (C) in the blood in the umbilical cord at the time of delivery. The levels reported for normal adult males are 0.85 ± 0.09 mg per 100 ml of plasma.

amino acids from the blood. Hence, when an amino acid is determined in the plasma after a fasting period of 14-16 hours, less may be found in the child than in the adult. (2) A difference in the absorptive capacity of children and adults might play some part in making plasma levels of the former lower, but this seems unlikely. Preliminary tests in this laboratory, involving analysis of the blood of previously fasted subjects after the ingestion of 0.1 gm of methionine per kg of body weight, have shown that the amount of methionine absorbed in children approximates that found in adults.

Conclusion. The concentration of apparent free *L*-methionine in the plasma of children is significantly lower than has been recorded in the literature for adult male subjects. The plasma of the blood in the umbilical cord at the time of delivery contains, on the average, the same amount of *L*-methionine as is recorded for adult males, and more than three times as much as is present in the plasma of "normal" children.

Sincere thanks are expressed to Doctor Harold Harper, University of San Francisco, for helpful suggestions.

16195 P

Sarcomas Induced in Rats by Implanting Cellophane.

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In the course of experiments performed on rats to produce hypertension by wrapping regenerated cellulose film (cellophane) around one kidney, the accidental observation was made that several rats developed sarcomas in the neighborhood of the cellophane. As it was known that spontaneous sarcomas are a rarity in this Sherman strain of albino rats it was decided to investigate this question as an independent problem.

Method. The cellophane employed was regenerated cellulose film, the particular sample used throughout these experiments being known as Visking 5-½ H.S. ("high-stretch") cellulose sausage casing. Two strains of albino rats were used, the Sherman and the Wistar. The rats were fed on a mixture of Purina Laboratory Chow and Rockland Rat Diet in pellet form.

Cellophane was introduced by two methods into 110 male rats, 8 to 10 months old. In 55 of these, the left kidney was loosely wrapped with cellophane according to the familiar method introduced by Page.[1,2] In the other 55 rats, a piece of cellophane 2-3 cm square was embedded subcutaneously in the abdominal wall, with a catgut suture passed through the corners to keep the film flat. At frequent intervals the site of the cellophane was palpated by an experienced technician to ascertain if any growth had started. The tumors were usually allowed to grow to a large size (2-5.5 cm in diameter) before the rats were sacrificed for histological examination.

Wherever possible, transplants were made from each tumor into 8-16 rats, usually males (but in some instances females), 6-8 months of age. A total of 422 rats were used for this purpose, transplantation being carried out in some instances to the third, fourth and even fifth generation. Transplants were made by inoculating fragments of tumor tissue (0.003 to 0.006 g) subcutaneously into the anterior abdominal wall by means of a hollow platinum-iridium needle.

Results. As in the original accidental observation, a number of tumors were induced at the site of insertion of the cellophane. More than 11 months were required for their development. In the perirenal series the shortest time taken for the production of a tumor was 362 days from the date of wrapping to the development of a tumor 8 mm in diameter, whereas in the subcutaneous series the shortest period for development was 471 days.

Consequently, those rats that died from other causes within 11 months from the date of cellophane insertion were excluded altogether from the statistics, since they did not live long enough to develop induced tumors.

Among the rats with wrapped kidneys only 23 survived over 11 months, and of these 8 (34.8%) developed large well-defined tumors. Of the 55 rats in which the cellophane was embedded subcutaneously 42 survived beyond 11 months, and of these 15 (35.7%) presented tumors. The diagnosis of all these tumors was confirmed by microscopic examination.

The tumors resulting from transplantation appeared after 7 to 175 days. A total of 199 tumors were produced by transplantation from the primarily induced tumors, but this includes 126 instances in which well-defined growths subsequently receded, making microscopic examination impossible.

Types of Tumor. Of the 23 primarily induced tumors there were 17 fibrosarcomas, 2 liposarcomas, one rhabdomyosarcoma, one

1 Page, I. H., *Science*, 1939, **89**, 273.

2 Graef, I., and Page, I. H., *Am. J. Path.*, 1940, **16**, 211.

undifferentiated sarcoma, one osteogenic sarcoma and one plasmocytoma. Metastases occurred in 3 instances. Among the tumors derived by transplantation there were 63 fibrosarcomas, and a few other miscellaneous growths. Variation in differentiation and growth rate was noted in different generations.

Discussion. We have arrived at no conclusion as to the nature of the carcinogenic agent in these experiments. Attention is called to the fact that the procedure described is one of the simplest known for the production of sarcomas. In this connection the production of rat sarcomas by Turner[3] by embedding disks of bakelite (phenol-formaldehyde) is of interest.

In the course of certain surgical procedures, cellophane has been used as a covering or sheath,[4] and left in the human body. We are not acquainted with any reports of the development of sarcoma in man subsequent to its use, but this possibility should not be forgotten.

Conclusions. 1. Sarcomas were induced in albino rats by the insertion of regenerated cellulose film either subcutaneously or by wrapping it around one kidney.

2. These tumors occurred in about 35% of the rats surviving the operation more than 11 months.

3. The tumors were transplantable.

4. This is a simple method for inducing sarcomas experimentally.

5. The foregoing results in rats should be taken into consideration in the surgical use of cellophane in man.

[3] Turner, F. C., *J. Nat. Cancer Inst.*, 1941, **2**, 81.

[4] Ingraham, F. D., Alexander, E., Jr., and Matson, D. D., *New Eng. J. Med.*, 1947, **236**, 403.

16196

Eugenol as a Stimulus for Gastric Mucous Secretion*†‡

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In the course of investigations in this laboratory on the characteristics of gastric mucus, various types of stimulation have been studied. These included subcutaneous injection of pilocarpine, gentle mucosal massage, and topical application of a number of aqueous solutions and emulsions including ether (saturated), ethyl alcohol (50%), isotonic NaCl (0.17 N), hypertonic NaCl (0.5 N), clove oil (5%), mustard oil (1%), and distilled water.[1,2,3] Among the several objectives of these investigations was the discovery of a mucus-stimulating agent which induces no parietal secretion and therefore might be adopted as a standard stimulus for further work on the physiology of mucus secretion. Of the nine stimuli already studied, the 5% clove oil-water emulsion was clearly the most suitable for the purpose, as evidenced by the following characteristics of specimens obtained after a single application of this agent: (a) high viscosity, (b) high pH,[4] and (c) a large total volume relative

* A preliminary report of this work was transmitted to the XVII International Physiological Congress (Hollander, F., and Lauber, F. U., *Communications XVII Internat. Physiol. Cong.*, 1947, p. 155).

† This investigation was conducted with the aid of grants from the Altman Foundation, and Wyeth, Inc.

‡ The authors wish to express their thanks to Dr. Sophya Lazard for conducting some of the experiments.

[1] Hollander, F., Lauber, F. U., and Stein, J. J., *Am. J. Physiol.*, 1947, **149**, 724.

[2] Hollander, F., and Stein, J. J., *Am. J. Physiol.*, 1943, **140**, 136.

[3] Hollander, F., Stein, J. J., and Lauber, F. U., *Gastroenterology*, 1946, **6**, 576.

[4] Hollander, F., *J. Nat. Cancer Inst.*, 1945, **5**, 367.

TABLE I.
Data for Several Characteristics of Gastric Pouch Mucus.
(Stimuli: aqueous emulsions of clove oil and eugenol.)

			Stimuli			
			Eugenol			
			Low concentration (¼-½%)	Intermediate concentration (1-2%)	High concentration (5%)	Clove oil, (5%)
Volume per exper.		Mean	1.7	4.1	6.4	2.9
		Stand. Dev.	1.89	3.75	3.15	1.19
		No. of exper.	13	21	65	9
pH		Range	4.35-8.60	4.76-8.87	7.53-9.22	7.28-8.47
		Mean	7.69	8.03	8.51	8.10
		Stand. Dev.	1.03	0.57	0.44	0.27
		No. of specimens	42	105	108	27
Consistency	No. (%) of specimens	Viscous	29 (64.4%)	64 (59.8%)	92 (64.8%)	27 (96.4%)
		Fluid	14 (31.1%)	30 (28.0%)	32 (22.5%)	1 (3.6%)
		Mixed	2 (4.4%)	13 (12.1%)	18 (12.7%)	0 (0.0%)
		Total	45	107	142	28
Opacity	No. (%) of specimens	Opaque	26 (59.1%)	54 (56.3%)	104 (81.9%)	26 (96.3%)
		*Non-opaque	18 (40.9%)	42 (43.7%)	23 (18.1%)	1 (3.7%)
		Total	44	96	127	27
Columnar Cells	No. (%) of specimens	Many	28 (68.3%)	76 (72.4%)	35 (83.3%)	19 (95.0%)
		†Few	13 (31.7%)	29 (27.6%)	7 (16.7%)	1 (5.0%)
		Total	41	105	42	20

* Non-opaque = transparent or translucent.
† Few cells = none or a small number of cells per field.

to that secreted spontaneously in the same period of time.

The one disadvantage in the use of clove oil as a standard stimulus arises from its being a mixture of several chemical compounds in variable proportions, rather than a single chemical individual. However, the chief component of this essential oil is eugenol (4-allyl-2-methoxy phenol) which comprises 82-87% of the mixture. Hence, it seemed that this compound may be responsible for a major part of the mucus-stimulating action of the clove oil, and, therefore, better suited for use as a standard mucus stimulus. We have investigated the secretory response of the gastric mucosa to topical application of aqueous emulsions of pure eugenol, and the results are presented in this report.

Procedure. The experimental technique was the same as that used previously for studying the action of clove oil and the other stimuli.[3] The eugenol emulsions were prepared in concentrations ranging from ¼ to 5%. Acacia (5%) was used as an emulsion stabilizer in the earlier experiments; later this was replaced by Tergitol Penetrant-4 (1/40%) with equally good results.§ Control experiments with these substances alone showed that they exert no mucus-stimulating action at these concentrations; the fluid obtained after their application was the same as spontaneously secreted mucus in appearance and rate of formation. As in our previous work, the eugenol stimulus was administered only after the pH of the control (pre-stimulation) specimens had risen above 6.0. In general, collection of the stimulated specimens was continued until the rate of secretion fell to its control value. The pH's were determined electrometrically, with a glass electrode, and were reproducible to ±0.02 of a unit. A total of 99 experiments (*i.e.*, stimulations) was performed with eugenol emulsions, using 9 dogs with Heidenhain pouches.

§ Tergitol Penetrants were kindly supplied by the Carbide and Carbon Chemicals Corp., New York City.

Results. The data on characteristics of mucus specimens obtained after eugenol and 5% clove oil emulsions are presented in Table I. Because of the small frequencies in some of the groups of data, the values for ¼ and ½% eugenol were combined into a single group designated "low concentration," and those for 1 and 2% into an "intermediate concentration" group. The resulting increase in the number of specimens in each group augments the chance of obtaining a significant statistical evaluation of the observations. The frequency of the 5% eugenol data being large, these were retained alone as the "high concentration" group.

Total volume of mucus secreted in response to a single application of stimulus. The mean volume of secretion per experiment increases with the concentration of eugenol in the stimulus, from 1.7 ml for the "low concentration" group to 6.4 ml for the "high concentration." This latter value, for the 5% eugenol concentration, is significantly greater‖ than that for 5% clove oil (2.9 ml per experiment). The datum for 1-2% eugenol ("intermediate" group) is also greater than that for 5% clove oil but the difference is not significant.

pH. As shown in Table I, the mean pH of eugenol-stimulated specimens increases with increasing concentration of the stimulus, the differences between the several groups being significant at or below the 1% level of probability.¶ Corresponding to this, there is an elevation of both the upper and lower limits of the range, but the data become increasingly homogeneous, as evidenced by a decrease in the range itself and in the standard deviation. The mean pH for 5% clove oil is significantly lower** than that of eugenol of the same concentration but is essentially the same as that for 1-2% eugenol. Since the 5% eugenol emulsion gives a higher mean pH and a lower standard deviation than any of the other stimuli which we have previously studied,[5] it is the most effective agent encountered to date for evoking an alkaline response from the stomach.

Consistency, opacity, and columnar cell content. Qualitatively, these physical characteristics of mucus were essentially the same for eugenol as for all the other topical stimuli, except mustard oil emulsion. Some of the specimens were distinctly fluid; others jelly-like or of intermediate viscosity. Some were transparent or translucent; others opaque. Some were cell-free or contained only cellular detritus; others contained many columnar cells—singly, or in ranks and clumps. In microscopic appearance, after being stained with toluidine blue, the eugenol-mucus smears were indistinguishable from those of clove oil-mucus. Numerically, the percentage incidences for the three categories of consistency shown in Table I are essentially the same for all 3 concentrations of eugenol; clove oil, however, gives a markedly higher percentage of viscous specimens. For opacity, the percentages of opaque specimens in the low and intermediate concentration groups are almost identical; whereas the value for 5% eugenol is considerably higher than these, and that for clove oil even greater.†† The values for columnar cell content show this same trend, although the differences are not significant statistically by the χ^2-test ($P>1\%$).

The consistently higher position of clove oil over eugenol, in regard to all three of these physical characteristics, is probably the result of the action of one or more compounds in the essential oil other than eugenol. In addition to the latter, clove oil contains vanillin, methyl alcohol, furfural, caryophyllene, acetyl

‖ This difference is significant at the 1% level of probability; $t = 3.3$, $n = 72$, $P = 0.18\%$.

¶ The difference between the mean-pH's for the low and intermediate concentrations gave the following statistics in the t-test: $t = 2.6$, $n = 145$, $P = 1\%$. For the intermediate and high concentration groups, $t = 6.9$, $n = 211$, $P < 0.01\%$.

** $t = 4.8$, $n = 133$, $P < 0.01\%$.

[5] Hollander, F., Lauber, F. U., and Stein, J. J., in preparation.

†† These differences in relative frequencies are statistically significant at the 1% level of probability, as shown by the χ^2-test. For the first of these—with categories of opaque and non-opaque specimens, and "high" and "intermediate" concentrations of eugenol—$\chi^2 = 17.4$, $n = 1$, $P < 0.01\%$. For the second—with categories of opaque and non-opaque specimens, and 5% clove oil and "intermediate" concentrations of eugenol—$\chi^2 = 14.8$, $n = 1$, $P = 0.01\%$.

eugenol, and eugenol acetyl salicylate,[6] and it is possible that some of these substances are particularly potent as desquamating agents, even in small quantities.

Summary. The characteristics of gastric mucous secretion, stimulated by topical application of aqueous eugenol emulsion in several concentrations, have been investigated on 9 Heidenhain pouch dogs. A 5% emulsion of clove oil, which had previously been found to be superior to all other mucus stimuli, was used as a basis of reference for determining the secretory value of eugenol. It was found that 5% eugenol yields larger volumes of secretion, with a higher pH, than the clove oil. The percentages of specimens possessing high viscosity, opacity, and columnar cell content are lower for the eugenol than for the clove oil. Since we have already cited reasons[3] for believing that *pure* gastric mucus is transparent, cell-free, and of variable consistency, it may be that the secretion yielded by eugenol differs less from pure mucus than does the fluid obtained with clove oil.

Five per cent eugenol emulsion is the most effective stimulus to mucus secretion which we have found to date—especially since it yields larger volumes of mucus which tend to have a higher pH than those induced by any of the other stimuli. The latter characteristic indicates also that eugenol has virtually no stimulating effect on the parietal cells. Furthermore, this substance is a pure compound, the major component of clove oil, whereas the latter is a mixture of at least 7 different chemical substances. Since the minor components of the essential oil may also exert some physiological effect on the mucosa, their absence from the pure eugenol enhances its value as a standard. Hence, we propose to adopt an aqueous emulsion of eugenol as a standard stimulating agent for further work on the physiology of gastric mucous secretion.

6 Wood, H. C., Jr., and Osol, A., *The Dispensatory of the United States of America*, 23° ed., Philadelphia, 1943.

16197

The Influence of Coramine on the Liver of the Young Rat.

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Coramine (nikethamide) is a compound which provokes a number of different pharmacological actions. It is capable of curing black tongue in dogs by virtue of its close structural relationship to nicotinamide;[1] it is often employed as a medullary stimulant; and it has a pronounced ability to increase the liver weight of young rats.[2] Coulson and Brazda[2,3] have presented evidence which indicates that the unsubstituted nitrogen of the heterocylic ring and the di-ethyl substitution of the amido nitrogen are both in part responsible for the liver enlargement which follows the administration of the compound.

In preliminary experiments the absolute liver weight increase caused by coramine by the end of a 28-day period could not be prevented by the inclusion of 1.2% methionine or 0.5% choline in the diet.[2] This weight increase suggested that the liver cells were being injured by the coramine and that this was followed by the very rapid regeneration which is seen in many types of liver injury. It was deemed desirable to design experiments

1 Smith, D. T., Margolis, G., and Margolis, L. H., *J. Pharm.*, 1940, **68**, 458.

2 Coulson, R. A., and Brazda, F. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 1.

3 Brazda, F. G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 19.

TABLE I.
Effect of the Oral Administration of Choline or Methionine to Young Rats on a 1% Coramine Diet.†

No. days	Rat final wt	Liver wt wet	Liver wt dry	Liver wt as % body wt	Liver fat, % wet wt	Liver fat, % dry wt	Liver, % solids	Gain in wt, g/day
				Control.				
5	63.9	3.05	0.97	4.80 ± 0.10*	4.83 ± 0.21*	15.11	32.23	+3.47
9	70.0	3.36	1.06	4.78 ± 0.09	4.22 ± 0.12	13.38	31.47	+3.30
14	99.7	4.78	1.54	4.79 ± 0.09	4.01 ± 0.27	12.41	32.10	+3.93
19	122.4	5.59	1.74	4.56 ± 0.10	3.52 ± 0.21	11.19	31.46	+4.03
24	132.4	6.10	1.96	4.60 ± 0.08	4.09 ± 0.14	12.94	31.93	+3.56
28	152.9	6.56	2.08	4.27 ± 0.09	4.27 ± 0.07	13.47	31.69	+3.85
				1% Coramine.				
5	52.2	4.32	1.49	8.31 ± 0.12	6.72 ± 0.54	19.82	34.14	+1.50
9	66.4	5.81	1.89	8.76 ± 0.19	7.66 ± 0.42	23.33	32.53	+2.56
14	83.8	6.87	2.38	8.25 ± 0.18	8.62 ± 0.50	25.19	34.33	+2.76
19	97.6	7.04	2.44	7.31 ± 0.25	7.88 ± 0.39	22.55	34.69	+2.71
24	106.4	7.85	2.70	7.38 ± 0.14	8.22 ± 0.71	23.29	34.58	+2.70
28	119.3	8.35	2.93	6.98 ± 0.17	8.60 ± 0.19	24.17	35.17	+2.73
				1% Coramine + 2.4% Methionine.				
5	39.1	3.09	0.94	7.94 ± 0.27	4.59 ± 0.19	15.06	30.45	—0.40
9	47.8	3.81	1.21	7.95 ± 0.14	5.19 ± 0.16	16.32	31.75	+0.26
14	63.1	5.32	1.80	8.33 ± 0.28	6.89 ± 0.87	20.89	33.48	+1.13
19	72.0	5.84	1.84	7.92 ± 0.17	5.40 ± 0.31	16.63	32.41	+1.41
24	84.7	6.22	2.04	7.44 ± 0.19	6.26 ± 0.52	18.88	32.45	+1.62
28	80.3	5.79	1.90	7.21 ± 0.10	5.49 ± 0.34	16.51	32.94	+1.34
				1% Coramine + 0.5% Choline.				
5	40.1	3.37	1.04	8.52 ± 0.21	3.98 ± 0.18	12.85	30.72	+0.76
9	53.9	4.06	1.36	7.59 ± 0.21	6.68 ± 0.42	19.76	33.16	+1.95
14	75.3	5.53	1.81	7.37 ± 0.24	6.17 ± 0.65	18.49	32.40	+2.53
19	82.9	5.92	1.85	7.16 ± 0.16	4.74 ± 0.18	15.33	30.89	+2.39
24	95.2	6.26	1.98	6.55 ± 0.19	4.58 ± 0.23	14.27	31.62	+2.42
28	109.0	7.32	2.41	6.66 ± 0.13	4.52 ± 0.23	13.66	32.84	+2.55

* P.E. $= \pm 0.6745 \sqrt{\frac{\Sigma(v)^2}{n(n-1)}}$

† Each figure in the table represents the average value obtained from 5 males and 5 females.

which would give some information on the nature of the increase in liver weight and which would help to explain the action of the compound.

Experimental. To determine the rate of the liver weight increase 4 groups containing 60 rats each were placed on a diet which consisted of 25% casein, 55% starch, 15% cottonseed oil and 5% salts.[4] Each rat also received a daily dose of one ml of Brewer's yeast extract (equivalent to one g of dried yeast) and one drop of Percomorph oil a week. The rats, weighing 35-45 g, were an inbred mixture of the Sprague-Dawley and Illinois strains. Coramine* was mixed into the diet at a level of 1% for all groups except the controls. One group received an additional supplement of methionine at a level of 2.4% of the diet and one the equivalent of a 0.5% choline supplement in the form of choline chloride. At intervals of 5, 9, 14, 19, 24, and 28 days, 5 male and 5 female rats, each from a different litter, were killed out of each of the 4 groups. The livers were weighed, the fat and water contents were determined by analysis and a specimen was saved for the preparation of histological sections. These were stained by the routine hematoxylin-eosin method. The water content was determined by drying the livers to constant weight in a vacuum desiccator over concentrated sulfuric acid at about 30 mm Hg pressure. The dried livers were then used for the determination of the fat (total lipid) content. They were extracted twice with hot alcohol, four times with an alcohol-ether mixture and ten times with ether. The solvents were evaporated and the fat was determined gravimetrically.

Both choline and methionine were added

[4] Hawk, P. B., and Oser, B. L., *Science*, 1931, **74**, 369.

* The commercial product in 25% solution was used.

TABLE II.
The Influence of the Ingestion of Coramine on the Liver Glycogen of Young Rats.

Group	No. rats	Liver wt as % body wt		% liver glycogen	
		Range	Avg	Range	Avg
Control	11	3.63-5.27	4.42 ± 0.09	1.40-3.75	3.09 ± 0.13
1% coramine	12	5.56-7.66	6.81 ± 0.11	1.66-3.28	2.39 ± 0.09

in amounts calculated to be far in excess of any normal requirements. Methionine in such a massive dose caused a considerable decrease in the growth rate and choline provoked a slight decrease (Table I). In each case the growth rate was in proportion to the food consumed. The comparatively high protein diet supplemented with methionine or choline was chosen in an attempt to provide dietary conditions such as to minimize any liver damage.

The increase in liver weight occurred in surprisingly short time and the livers remained larger than in the controls for the full 28-day period in all of the three experimental groups (Table I). There was a gradual decline in liver wt/body wt ratio in the last 2 weeks in all 3 groups. It is evident that neither choline nor methionine is capable of modifying the increase in the liver wt/body wt ratio caused by coramine. The slight differences noted in the three groups may be explained by the decreased fat content in the groups receiving choline or methionine.

Those rats which received coramine had livers which showed some elevation in the fat content as early as 5 days (Table I) and continued to show roughly a constant level of fat for the full 28-day period. Both choline and methionine reduced the fat content of the liver to a level significantly below that of the coramine rats but slightly above that of the controls. The solid content of the livers was the same in all 4 groups. This becomes evident if one subtracts the excess fat in the coramine series prior to calculation. The absolute increase in liver weight must be due to an increase in both solids and water in the same proportion as that found for the control series.

Since the increase in liver weight was not due to a disproportionate increase in water or fat, experiments were devised to determine whether this might be due to glycogen. Eleven rats, 7 females and 4 males, were placed on the control diet. Twelve rats, 7 females and 5 males, were placed on the 1% coramine diet. At the end of 6 days both groups of rats were killed and the liver glycogen was determined by the method of Good, Kramer and Somogyi.[5] It was decided that a longer period was unnecessary (Table I). The results appear in Table II.

It is evident that the glycogen content of the livers taken from the coramine treated rats, although somewhat lower, was not seriously different from that of the control rats. Glycogen storage was not responsible for the liver enlargement. The increase in liver weight in the glycogen series was less marked than in the series reported in Table I. This discrepancy may possibly be explained by the fact that this experiment was conducted during the hottest time of the year. The general phenomenon of significant liver enlargement, however, is still apparent.

Microscopic examination showed that the livers from rats receiving coramine had intracellular fat globules. Close correlation existed between the histological picture and the gravimetric analysis for fat. A prominent feature of the liver parenchyma was the large number of mitoses and binucleate cells. There was a slight increase of interlobular connective tissue in the livers of the experimental animals. Aside from the significant decrease in fat in the livers of rats receiving coramine plus choline or methionine no other effects due to these lipotropic agents were observed. Livers from rats after the 28-day experimental period were little different from those of the 5-day groups.

Discussion. In spite of the fact that cora-

[5] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

mine seems to promote a very rapid "regeneration" of liver tissue, the liver shows relatively little damage even after 28 days on a coramine diet. It is quite possible that necrosis or cirrhosis or both might have been produced by continuing the experiment for several months. Blumberg and Grady[6] found that over 300 days were required to produce fibrosis in rats with fatty livers.

It is surprising that the full effects of coramine on the liver wt/body wt ratio are manifest in 5 days or less and that no obvious progressive change occurs from then up to the 28th day. The growth rate was depressed, but not seriously, and all of the 180 experimental animals appeared to be in good condition at the time of autopsy.

Essentially none of the results presented, either chemical or histological, indicate any serious abnormality in the liver parenchyma of the coramine treated rats. A hypothesis as to the nature of the action of coramine on the liver cannot, however, be advanced. The rapidity and degree of enlargement of the liver superficially resembles the effect produced in organs by their respective trophic hormones. Whether this resemblance is more than superficial remains to be determined.

Summary. (1) Coramine produces a great increase in the liver wt/body wt ratio in growing rats. (2) This increase which is manifest within 5 days or less and which is still apparent for at least 28 days cannot be prevented by the ingestion of choline or methionine. (3) The percentage of water and glycogen in these livers is essentially the same as in the controls. Coramine causes some elevation in the fat content which may be prevented by the administration of choline or methionine. (4) On microscopic examination, aside from the fat globules observed in the livers of rats receiving coramine alone, no marked pathological changes were evident. Binucleate cells and mitoses were present in large numbers.

The authors wish to express sincere thanks to Dr. T. Hernandez for carrying out the microscopic examination of the histological sections.

6 Blumberg, H., and Grady, H. G., *Arch. Path.*, 1942, **34**, 1035.

16198

Growth of Animal Tissue Cells in Artificial Media.*

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Multiplication and growth of tissue cells *in vitro* depend on the presence of two kinds of substances in the culture media. Substances of a protein nature present in extracts from embryonic tissues were shown by Carrel[1] to act as growth promoting factors. These high molecular weight substances are present only to a very limited extent in blood plasma or serum. Attempts at the isolation of these very labile substances, grouped by us under the name "embryonin",[2] have shown that they can be purified by the extraction methods outlined by Hammarsten[3] for the isolation of a nucleo-protein. Other methods of purification have so far failed. These results have been confirmed by Davidson and Waymouth.[4]

When the culture media (plasma, serum, embryo extract) are dialysed against Ringer-glucose solution, and Tyrode's solution is added (furnishing inorganic phosphate and bicarbonate), the tissue cells disintegrate

* Aided by grants from Rask-Orsteds Fond and from Kong Christian den Tiendes Fond.

1 Carrel, A., *J. Exp. Med.*, 1913, **17**, 14.

2 Fischer, A., and Astrup, T., *Pflügers Arch.*, 1943, **247**, 34.

3 Hammarsten, E., *Z. physiol. Chem.*, 1920, **109**, 141.

4 Davidson, J. N., and Waymouth, Ch., *Biochem. J.*, 1945, **39**, 188.

within 24 hours, even in the presence of large amounts of dialysed embryo extract containing embryonin as a growth promoting factor.[5] Evidently some low molecular weight substances essential for the metabolism of tissue cells have been removed by the dialysis. The action of these diffusible substances differs from the action of embryonin, for the diffusible factors are able to maintain the cells in good condition, though in the absence of embryonin no growth is obtained. Embryonin, on the other hand, acts only in the presence of the diffusible substances; it does not maintain the cells in dialysed media. For this reason, we call embryonin a growth promoting factor, while for the diffusible substances of low molecular weight we use the term accessory growth factors. These two terms are used throughout in order to distinguish between the two types of substances and between their different modes of action.

Preparations of accessory growth factors that induce the growth of tissue cells placed in dialysed media (containing embryonin) have been obtained from blood, serum, kidney, heart, yeast, and barley malt.[6] The partial purification of malt extracts[7] showed that it is possible to remove considerable quantities of inactive material from these mixtures without any significant interference with the biological properties of the extract, which suggests that only a few of the substances present may be responsible for its action on tissue cells. Recently, we were able, by a treatment with living bakers' yeast, to remove from such an extract all the fermentable carbohydrates. With this product, we were able to show the importance of glucose, mannose, and fructose in cell metabolism.[8] The further purification was only partially successful, but from the diffusible substances present in calf embryo muscle we obtained very potent preparations.[9]

Accessory Growth Substances in Dialysates from Embryo Juice. In these experiments, the test material consisted of chicken heart fibroblasts cultivated for 5-8 passages in the usual manner before being placed in the dialysed media in Carrel flasks. The technic has been described previously.[5,6,7] The crude material is prepared as follows:

The muscles are removed from 5 calf embryos, passed through a meat chopper, and an equal volume of Ringer's solution is added. After standing for about one hour at room temperature, with occasional stirring, the mixture is pressed through gauze. The resulting extract (8 liters), corresponding to 4 liters of muscle, is dialysed in cellophane tubings against 2 portions of 8 liters each of distilled water for 2 days. Toluene or xylene must be added in sufficient quantities as preservatives, otherwise inactive products are obtained. The combined dialysates containing about 75% of the dialysable substances of the extract are evaporated almost to dryness *in vacuo* in a water bath at 40-50°. The residue is treated with 120-150 ml of a mixture of 1 vol. glacial acetic acid and 2 vol. of methanol (possibly with slight heating) and, as a result, most of the inorganic salts (chlorides) are left undissolved, while all the active substance is found in the solution. After centrifugation, it is precipitated with 3 vol. of absolute ethyl alcohol, and the precipitate is isolated by centrifugation or filtration on a sintered glass filter and treated with absolute alcohol and dry ether. The product is very hygroscopic and must be treated with utmost care at this point. It is immediately dried *in vacuo* over H_2SO_4 and solid NaOH in a desiccator. When dry, it can be kept indefinitely in a stoppered bottle. Preparation V-521 yielded 32.4 g, corresponding to 10.8 mg substance per ml of embryo muscle. It contained 3.44% N, 1.37% inorganic P, and 1.41% organic P. Addition of 0.2 ml of a solution containing 10-33 mg of this product per ml (in physiological saline, neutralized and sterile-filtered) to a Carrel flask containing dialysed media restores the ability of the media to promote the normal appearance and growth of the tissue cells.

5 Fischer, A., *Acta Physiol. Scand.*, 1941, **2**, 143.

6 Fischer, A., and Astrup, T., *Pflügers Arch.*, 1942, **245**, 633; Astrup, T., Fischer, A., and Volkert, M., *Acta Physiol. Scand.*, 1945, **9**, 134.

7 Astrup, T., and Fischer, A., *Acta Physiol. Scand.*, 1945, **9**, 183; 1946, **11**, 187.

8 Astrup, T., Fischer, A., and Oehlenschläger, V., *Acta Physiol. Scand.*, 1947, **13**, 267.

9 Astrup, T., Fischer, A., Ehrensvärd, G., and Oehlenschläger, V., *Acta Physiol. Scand.*, in press.

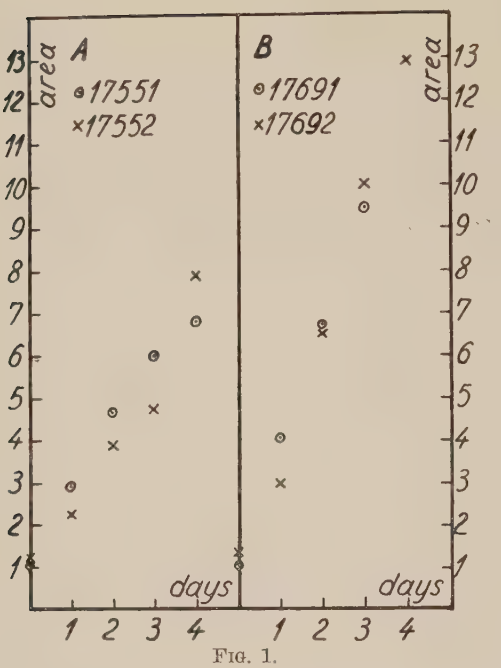

FIG. 1.

A. Effect on tissue cells of soluble calcium salts prepared from calf embryo dialysate after removal of inorganic phosphates. Culture No. 17551: Dry preparation isolated from dialysate (V-521-1). Culture No. 17552: Soluble Ca-salts (V-521-61). (Abscissa: Days. Ordinate: Relative growth area.)

B. Effect on tissue cells of a calf embryo dialysate after treatment with yeast and removal of inorganic P. Culture No. 17691: Soluble Ca-salts before yeast treatment (V-521-61). Culture No. 17692: Soluble Ca-salts after yeast treatment (V-521-74).

With barium acetate it is possible to precipitate all the active substances together with all the phosphorus. After dissolving this precipitate and removing the Ba^{++}, all inorganic P may be precipitated with Ca^{++} without significantly decreasing the activity.

A dry preparation, V-537-1, prepared as described, contained 0.227 mg N, 0.1290 mg inorganic P, 0.0406 mg organic P, and 2.50 mg sugar per ml solution.

1.48 g of V-537-1 (corresponding to 200 ml original solution) are dissolved in 20 ml water, and 20 ml ethanol is added. After making the solution just alkaline to phenolphthalein with 2N NaOH, 2 ml 30% Ba-acetate solution are added. The precipitate is removed by centrifugation, dissolved in water by means of diluted HCl, Ba^{++} is removed with sodium sulphate, the centrifugate diluted to 200 ml, made alkaline to phenolpththalein with 2N NaOH, and 4 ml 10% $CaCl_2$ solution, saturated with $Ca(OH)_2$, is added. The solution is neutralized, concentrated *in vacuo* to 50 ml (4 times the original concentration), sterile-filtered and tested as usual. The solution is almost as active as the original product, and contains, per ml of the original concentration, 0.053 mg N, no inorg. P, 0.0246 mg org. P, and 0.56 mg sugar (preparation V-537-11).

The mother liquor from the Ba-acetate precipitation and the calcium precipitate were both almost inactive (Fig. 1 A).

By treatment with yeast, it is possible to remove part of the sugar in the preparations, and, at the same time, the organic phosphates are almost completely transformed into inorganic phosphates and may be removed as such by treatment with $CaCl_2$. These operations do not interfere significantly with the biological action of the preparations. Thus, preparation V-521-74 contained originally, per ml, 3.70 mg sugar, 0.190 mg inorg. P, and 0.161 mg org. P. After treatment of 200 ml with 10 g of bakers' yeast, overnight at room temperature, and removal of inorg. P with $CaCl_2$-solution at pH 8-9, there remained 1.34 mg sugar per ml but no inorg. or org. P. Fig. 1 B shows the activity tested in 4 times the original concentration.

Synthetic Accessory Growth Substances. The first attempt of any significance to devise a synthetic medium was made by Baker and Ebeling.[10] But because their results were based on the use of a digest of whole blood, they are of little use in disclosing the mechanism of cell nutrition, even though a medium made in this manner may be useful for other purposes.

The inability of embryo juice alone to furnish the substances necessary for normal growth of tissue cells was clearly demonstrated by Baker[11] who showed that serum is needed to furnish additional nutriment. Work on a synthetic medium must proceed, therefore, along 2 lines, for substances of both high and low molecular weight are involved.

[10] Baker, L. E., and Ebeling, A. H., *J. Exp. Med.*, 1939, **69**, 365.

[11] Baker, L. E., *J. Exp. Med.*, 1939, **69**, 625.

TABLE I.
Mixture V-605 of Biologically Active Substances Tested on Tissue Cultures.
(Mg of substances contained in 1 liter solution.)

$NaCl$	7500	*l*(—)-Lysine, 2HCl	15
KCl	200	*l*(—)-Histidine, HCl	5
$CaCl_2$	200	*l*(—)-Arginine	2
$MgCl_2$	100	*d,l*-Valine	14
Na_2HPO_4	50	*l*(—)-Leucine	9
$NaHCO_3$	1000	*d,l*-Isoleucine	10
		d,l-Threonine	12
$FeCl_2$	0.6	*d,l*-Phenylalanine	7
$CuCl_2$	0.2	*l*(—)-Tryptophan	2
$MnCl_2$	0.3		
$ZnCl_2$	1.0	*d,l*-Methionine	6
$CoCl_2$	0.01	Choline (as hydrochloride)	10
		Creatine	10
Glucose	800	Nicotinic acid	0.3
Mannose	100		
Galactose	100	Cystine	5
Inositol	20	Glutathione	5
Adenosinetriphosphate	200	Pantothenic acid	0.07
Fructose-diphosphate	100	Biotin	0.007
β-Glycerophosphate	100		
Inosinic acid	30	*p*-Aminobenzoic acid	1
		Hypoxantine	100
Cozymase	5		
Thiamine	3	Sodium succinate	10
Riboflavine	0.2	" fumarate	10
Pyridoxine	0.3	" malate	10
		" oxaloacetate	10
Glutamine	250		
		Ascorbic acid	2
		Methylnaphtohydroquinone-sulphate	0.005

Among the substances of high molecular weight are the growth-promoting substances of protein nature contained in the embryo tissue juice, the presence of which are necessary for cell multiplication. Cell survival, however, is possible without this addition. Further, the proteins of serum may play a role that has not yet been clarified. And then there are the substances of low molecular weight contained both in the embryo tissue juice and in the serum.

By dialysing the media, as we do, the problems are simplified, for we retain all the high molecular weight substances and have only to substitute the low molecular weight, dialysable substances by a synthetic mixture. We feel that it is necessary to distinguish clearly between the effects of the dialysable and of the non-dialysable substances and to solve these two questions separately. Further, we always add to our media a surplus of dialysed embryo tissue juice in order to furnish the growth-promoting factor and thus to obtain active growth of the cells. In this manner, we have a much more rigid proof of the ability of the added mixture to provide accessory growth substances than is the case when only the life of the cells is maintained. In this respect, our investigations differ from those of most previous authors.

We next proceeded to devise a mixture of all the substances of biological importance known to be present in animal tissue, and undertook to use them in concentrations comparable with those found in the living organism. In this manner, we devised Mixture V-605, described in Table I. The mixture was made from neutralized concentrated sterile solutions of the various groups of components, and 1 ml was used in the medium in a Carrel flask. Tested in the manner described, very intense growth and normal appearance of the cells were obtained even for cultures of small size. In the absence of glutamine, the mixture was rather active when tested on large cultures, but almost inactive on small cultures. Glutamine alone was quite inactive. Removal of the group containing the organic phos-

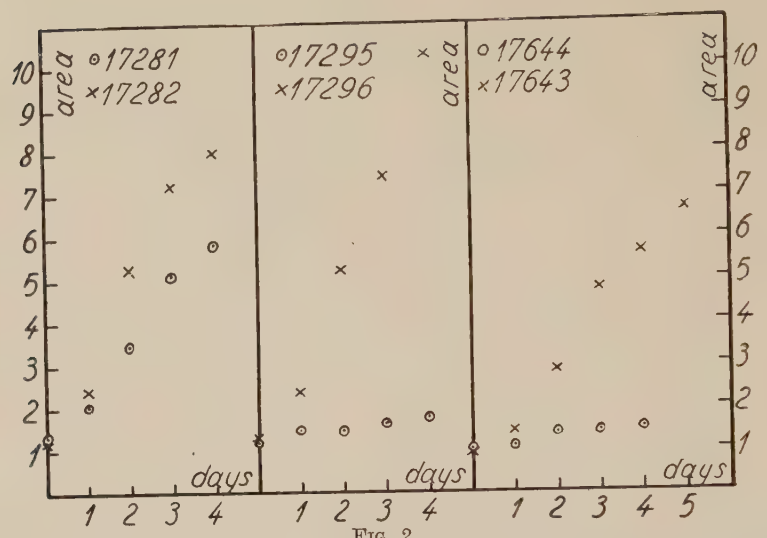

FIG. 2.

Effect of synthetic mixture V-605 on tissue cultures. Culture No. 17281: Mixture V-605 without glutamine. Culture No. 17282: Mixture V-605. Culture No. 17295: Glutamine alone. Culture No. 17296: Mixture V-605. Culture No. 17643: Mixture V-605. Culture No. 17644: Mixture V-605 without organic phosphates.

phates yielded a quite inactive mixture. Examples are shown in Fig. 2. Most of the other substances, however, could be discarded without influencing the action of the mixture on the cells, and in this manner we arrived at mixture V-612 (Table II), in which we have further increased the concentrations of organic constituents, except glutamine, with good results. Fig. 3 shows the action of this mixture with and without glutamine, tested on comparatively large cultures. The effect of glutamine is evident. The concentration of glutamine could be lowered to one-tenth without significantly interfering with its supplementary effect on the mixture. Further experiments show that it is possible also to discard β-glycerophosphate and inosinic acid, thus retaining only fructose-diphosphate (Fig. 3). Removal of the amino acids significantly decreases the activity. The importance of each individual amino acid has yet to be studied.

Discussion. A comparison of Tables I and II shows the substances not essential for growth of tissue cells under the conditions of the experiments. They include the heavy metals, known for their catalytic action on certain enzyme reactions, adenosine-triphosphate, cozymase, the different vitamins tested, choline, creatine, and the C_4-acids (functioning in the Krebs

TABLE II.

Simplified Mixtures V-612 and V-614 for Use with Tissue Cultures.
(Mg of substances contained in 1-liter solution.)
(β-glycerophosphate and inosinic acid were omitted in mixture V-614.)

	Mg
NaCl	7500
KCl	200
$CaCl_2$	200
$MgCl_2$	100
Na_2HPO_4	50
$NaHCo_3$	1000
Glucose	2000
Fructose-diphosphate	200
(β-Glycerophosphate	200)
(Inosinic acid	60)
l(—)-Lysine, 2HCl	30
l(—)-Histidine, HCl	10
l(—)-Arginine	4
d,l-Valine	28
l(—)-Leucine	18
d,l-Isoleucine	20
d,l-Threonine	24
d,l-Phenylalanine	14
l(—)-Tryptophan	4
Cystine	10
Glutathione	10
Glutamine	250

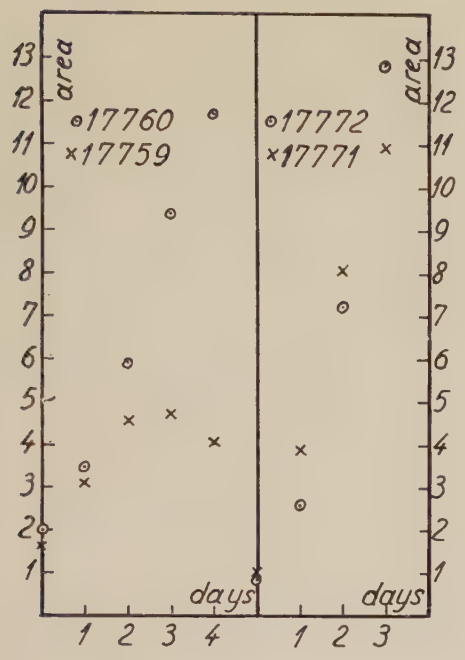

FIG. 3.
Effect of synthetic mixtures V-612 and V-614 on tissue cells. Culture No. 17759: Mixture V-612 without glutamine. Culture No. 17760: Mixture V-612. Culture No. 17771: Mixture V-612. Culture No. 17772: Mixture V-614.

cycle). It was surprising to us that the tissue cells would show normal growth in the absence of several of these substances. It may be that during the relatively short growth period (5-8 days) the constituents of the original tissue fragment suffice for the metabolism of the tissue cells, or that some of the components otherwise considered to be low molecular weight substances may be retained in the dialyzed plasma in combination with proteins. It is known, for example, that the heavy metal ions and certain organic components, *e. g.*, vitamin B_6, may be separated from proteins only with difficulty.

The vital importance of glutamine was expected in view of the relation between its function as a growth factor for certain streptococci and its role in streptococcal glycolysis, as found by McIlwain.[12] The importance of fructose-diphosphate is well understood in relation to its action in fermentation, and it may furnish a sufficient amount of hexose-diphosphate for the glycolytic system during the first hours after transplantation of the tissue fragment and before the cells themselves are able to regain all their normal enzymatic functions. It is important in this connection, however, to remember that it seems possible to remove all organic phosphorus from the previously described accessory growth substances contained in dialysates from embryo tissue juice without interfering with the biological activity. These products contain no glutamine and their mode of action may be different from that of the synthetic mixture described here.

Recently, White[13] has attempted to devise a completely synthetic medium. Our results are not in accordance with those of previous authors, and especially not with those of White, and though we consider it too early to discuss these differences a few points may be stressed. First of all we use a different technic, which in our opinion simplifies the problems in question by making possible a stepwise solution of the very complicated phenomenon of tissue growth. It is of special importance that while previous authors have preferred to work with media intended for maintenance of the cells for long periods, we use the active growth of cultures over short periods as a measure of the ability of the media to furnish the necessary substances. In this respect, it is of interest to note that two of our most important substances, *i. e.*, glutamine and fructose-diphosphate, are absent from the mixture described by White. Also, though White found that fibroblast migration is very much more active during the first few days in dextrose than in sucrose, he considers it probable that sucrose is superior to dextrose if the nutrient is not to be renewed frequently. In our experience, the tissue cells are completely unable to metabolize sucrose (saccharose).[8] We suppose, therefore, that the tissue cells in White's experiments use the carbohydrates (dextrose) contained in the tissue pulp used for cultivation, and that the cells of the pulp would survive equally well if no sucrose at all were added. White seeks to avoid unknown constituents in the culture media (*e. g.*, serum, peptone, fibrin-digest, tissue extract, and sim-

[12] McIlwain, H., *Biochem. J.*, 1946, **40**, 67, 460.

[13] White, P. R., *Growth*, 1946, **10**, 231.

ilar products), but in the pulp of macerated tissues used for cultivation he introduces large amounts of cell constituents of both high and low molecular weight. They may be removed by repeated washings either before or after they are placed in the culture tubes, and thus the difference between his results with dextrose and sucrose may be explained. The active growth of his cultures of tissue cells indicates the presence of the growth-promoting substance "embryonin" in his media, this substance being present in the macerated cells. A comparison with controls consisting of cultures made from washed tissue pulp would have been desirable.

Summary. 1. The difference between growth-promoting substances and accessory growth substances and between their different modes of action is pointed out. 2. From dialysates from embryo juice, preparations may be obtained which completely restore the ability of the dialysed culture media to induce normal growth of the tissue cultures. They may be prepared free from organic phosphates and glutamine. 3. A synthetic mixture is described which has the same properties of restoring the dialysed media. The most important of its components seem to be glutamine and fructose-diphosphate.

16199

Hereditary Dwarfism in the Descendants of Mice Receiving Methylcholanthrene—Parallel Induction.

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The following biological variants have been obtained in the descendants of mice whose ancestry have been injected with methylcholanthrene for many generations. (1) Several germinal mutations changing susceptibility to tumors induced at the site of the injection of the carcinogen. (2) Germinal mutations altering the incidence of specific types of tumors, following the injection of the methylcholanthrene and subsequently under spontaneous conditions in their untreated descendants—such as bronchiogenic carcinoma, leiomyo-sarcoma of the uterus, and gastric lesions of several histological types. (3) Many embryological disturbances such as dextrocardia, dextroversion, situs inversus, anovariae, absence of the cervix and vagina, absence of one kidney together with the absence of the horn of the uterus on the same side, craniorachichisis, several eye lesions, and possibly (?) twins. (Twenty-two pairs of twins have been obtained in mice which have been injected with methylcholanthrene. In view of the reported rarity of this condition in mice (only three cases could be found in the literature) it would seem that twinning in these methylcholanthrene-treated mice is highly significant. However, in more recent work especially by W. F. Hollander,[1] it has been found that twinning or placental fusions in mice are of very frequent occurrence. It is doubtful, therefore, whether the injected methylcholanthrene had much influence on the development of this biological variant.) The above biological variants are considered to be induced by methylcholanthrene when they have fulfilled one of the following criteria: either (a) they have occurred only among the injected mice or their immediate descendants and never among a comparable group of controls, or (b) they have occurred among the methylcholanthrene-treated mice at an increased frequency over the controls. (4) Several physiological and morphological charac-

* This experiment has been made possible by grants from The Jane Coffin Childs Memorial Fund for Medical Research, The Anna Fuller Fund, and The American Cancer Society, Committee on Growth.

[1] Hollander, W. F., unpublished data.

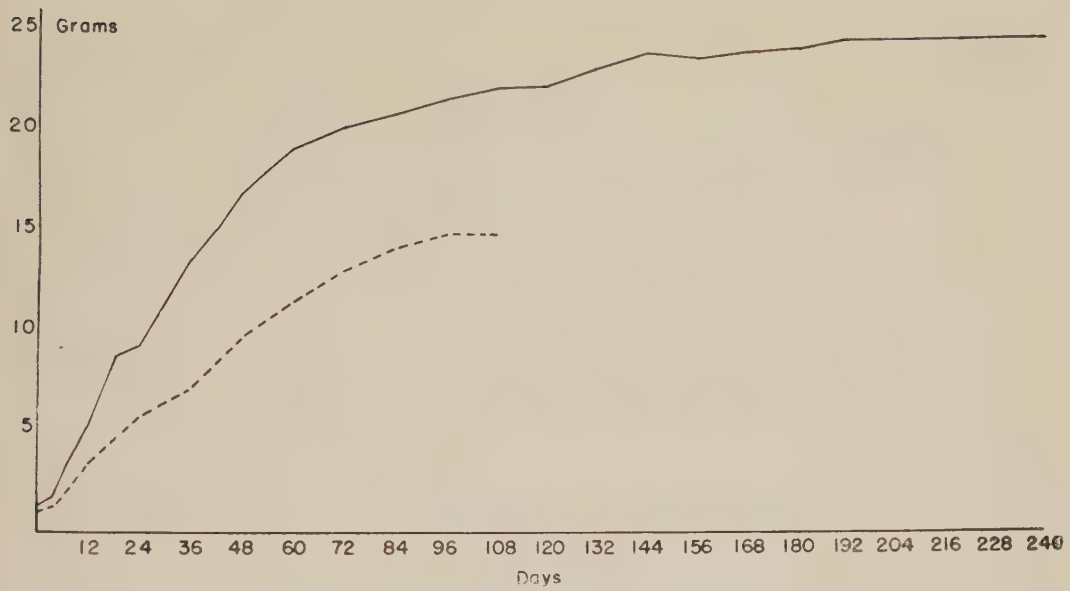

CHART 1.

This chart presents data on the growth rate of (a) normal male mice of the pBr subline of the NHO stock (solid line) and (b) hereditary dwarf mice of the same stock (short dash line). Time in days is given on the base line, body weight in grams is given on the vertical line.

teristics such as precocious opening of the vagina, delayed opening of the vagina even up to one year of age, and complete imperforate vagina, increased fecundity, precocious sexual maturity, increased fertility, excessively large first litters, rapid growth rate with the production of giant mice weighing up to 86 g of weight, and precocious and complete sterility. (5) Numerous cases of somatic mosaics especially involving hair pigmentation, and (6) the production of many germinal mutations involving hair color, eye color and distributional patterns of pigmentation.[2-8]

Recently Carr[9] has reported 7 biological variants produced in mice by the subcutaneous injection of 1:2:5:6-dibenzanthracene. He reports 1. Hydrocephalus, 2. Absence of left horn of the uterus, 3. Brain hernia, 4. Brown, 5. Pink eye, 6. Recessive spotting, and 7. Chinchilla. The last 4 of these variants were proven to be genetic recessives.

It is a well recognized fact that the carcinogens are inhibitory in action.[10] Thus when one injects a mouse with one of the carcinogens the normal growth rate is inhibited. In the pBr subline of the NHO descent whose ancestry has been injected with methylcholanthrene for many generations and the mice selected toward induced cancer resistance, the mice very seldom weigh more than 25 g when fully grown. The normal growth rate for mice of this subline is given on the solid line of Chart 1. On the 31st of March, 1947, it was noted that a pair of these pBr mice had produced a litter of young in which there were 4 normal and one dwarf mouse. These mice and their close relatives have produced, to date, a total of 121 mice in 21 litters in which, at least, one dwarf has appeared. Of these 121 mice, 92 were normal and 29 dwarfs. On a 3:1 basis one would expect 90.6 normals to 30.2 dwarfs. Thus it is obvious that dwarfism is a simple mendelian recessive.

2 Strong, L. C., *Arch. Path.*, 1945, **39**, 232.

3 Strong, L. C., *J. Nat. Cancer Inst.*, 1945, **5**, 339.

4 Strong, L. C., *Nat. Acad. Sciences*, 1945, **31**, 290.

5 Strong, L. C., *Yale J. Biol. and Med.*, 1946, **18**, 145.

6 Strong, L. C., *Yale J. Biol. and Med.*, 1946, **18**, 359.

7 Strong, L. C., *Science*, 1946, **103**, 554.

8 Strong, L. C., *American Naturalist*, 1947, **81**, 50.

9 Carr, J. C., *Brit. J. Cancer*, 1947, **1**, 152.

10 Boyland, E., Edgar Allen Memorial Lecture, New Haven, Conn., Oct. 8, 1947.

The growth rate for dwarf mice up to 108 days of age is given on the short dash line of Chart 1.

The dwarfs are smaller in size than the controls at birth and appear to be restless. They deviate strikingly from the controls at 2 to 3 days of age and, in competition with their normal siblings, they have invariably died before the weaning age of 30 days. A few days before death they lose weight progressively. However, when their normal siblings have beeen removed from the mother, the dwarfs will live for a longer time. So far only 5 have reached 100 days of age. Of these one pair has reached sexual maturity and has produced 16 young in 2 litters—all of which were dwarfs. One of these young was also a waltzer but unfortunately died before reaching sexual maturity, so that the biological nature of this waltzing could not be determined.

It is perhaps more than a coincidence that the mutations reported by Carr are the same as have been obtained in my laboratory. All 4 of his mutants have occurred several times in the descendants of mice which have been injected with methylcholanthrene. In addition, however, I have obtained many other germinal mutations, notably three types of dominant spotting, etc. One may entertain the possibility that perhaps there may be a specificity of action of the inducing system upon the genetic mechanism, an idea that has been mentioned previously. It must be borne in mind, however, that the first mutations that have been induced with methylcholanthrene and with 1:2:5:6-dibenzanthracene have been at loci which have given rise to spontaneous mutations in the past—thus indicating perhaps a greater mutability than other loci in the germ plasm of mice. The greatest difference between Carr's data and my own is, apparently, in the greater frequency of dominant mutations in my series. For example, with methylcholanthrene the recessive gene b has mutated to dominant B eight times, whereas the gene B has only mutated to b once. It is also clear that both non-genetic and genetic biological variants are being produced in separate laboratories. In mice, many biological variants occur for which no genetic evidence of inheritance can be obtained. It is consequently better to use the term biological variant until evidence of inheritance can be established. When this evidence is forthcoming the variant thus becomes a mutant. Among the most interesting non-genetic variants are the somatic mosaics being induced with methylcholanthrene. The fact that both methylcholanthrene and 1:2:5:6-dibenzanthracene are powerful carcinogens and that consequently the experimental animals are giving rise to a multiplicity of cancers should not be lost sight of.

Parallel induction maintains that an induction system may influence both the soma and the germ plasm—perhaps conditioned by some biological bond between the gene and some characteristic of the soma conditioned by gene action. This appears to be the case in the induction of gastric lesions under the influence of methylcholanthrene. This gastric lesion became hereditarily established by a mutation on the brown tagged chromosome.[5] The production of hereditary dwarfism in mice seems to be a second case of parallel induction. That is, that the induction system of methylcholanthrene or one of its metabolites has inhibited the normal growth rate of mice for several generations. When a germinal mutation took place it took place in the direction of the inductive influence. However, this has not always been the case. For example, the giant mice referred to previously apparently originated by some biological change (perhaps germinal) which was counter to the trend of biological induction.

The embryological disturbances may be brought about, perhaps, by an inhibitory action of the carcinogen at a critical period of the development of the embryo. For example, the absence of the cervix could be explained by the fact that perhaps the distal end of the Müllerian duct did not develop normally. The carcinogen is also inducing mutations and at the same time bringing about changes in the soma leading toward neoplasia. This multiplicity of biological effects by the injection of a pure compound may eventually be better understood when the metabolism of the carcinogen is more fully understood.

Summary. Among the germinal mutations

induced in mice following the subcutaneous injection of methylcholanthrene for many generations is hereditary dwarfism. This condition is inherited as a recessive. The relation of this mutation to the general problem of parallel induction is briefly indicated. Thus it is becoming more obvious that both non-genetic and genetic biological variants are being produced in mice by methylcholanthrene. The mechanism involved may be different for the different variants obtained but perhaps there may be a common denominator in them all.

16200

Effect of Muscular Fatigue on Histamine-Provoked Ulcer with Observations on Gastric Secretion.

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Complete physical rest is frequently mentioned by internists as a valuable adjunct in the medical management of peptic ulceration. This precept has been derived primarily from clinical observation as there are few if any experimental studies in recent medical literature which deal with the effects of muscular fatigue on alterations in gastrointestinal tract function particularly as they pertain to the ulcer diathesis.

It is the purpose of this report to indicate our observations on the effect of moderately severe muscular fatigue on the incidence of the histamine-provoked ulcer and upon gastric secretion in dogs.

Method of Study. Experiment 1. Fifteen healthy, dewormed dogs weighing from 26 to 54 lb, of young to medium age, were run from 3 to 4 hours consecutively each day in the morning on 5 to 6 successive days on an electrically driven endless belt treadmill. The dogs were allowed a 10-minute rest period each hour and water *ad lib.* The treadmill speed was calculated at 1.5 to 2.0 miles per hour which amounts to a brisk walking pace for the average sized dog. The slope of the treadmill was varied between an incline of 5 to 20° from horizontal depending on the dogs' physical condition. Just prior to being placed on the treadmill each day, the dogs were given an injection intramuscularly of 30 mg of histamine base-in-beeswax mixture prepared after the method of Code and Varco.[1] The dogs were fed a diet of horsemeat, milk, dog biscuit each day after finishing their period on the treadmill. Rectal temperatures were taken before the start of the exercise and after each hour of exercise as a gross indication of the degree of muscular effort.

As a control series, 13 animals of comparable size, age, and health were fed and injected daily with histamine as above, but were not run on the treadmill. In addition 2 dogs were run on the treadmill as above, but were not given histamine. In these 2 animals the effect of the muscular exercise on hemoglobin, hematocrit, leukocytes differential count, and blood sugar was studied. All animals were sacrificed with examination of their gastro-intestinal tracts at the end of their test period of 5 to 6 days on the treadmill and/or 5 to 6 consecutive daily intramuscular histamine-in-wax injections.

Experiment II. Studies on gastric pouch secretion were made using 2 Heidenhain-type pouches made over one year prior to the onset of these experiments. Both animals used were

* National Cancer Trainee. This work is supported in part by the U. S. Public Health Service, the Augustus L. Searle Fund and the John and Mary R. Markle Fund for Surgical Research.

1 Code, C. F., and Varco, R. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 475.

TABLE I.
Production of Ulcer in Dogs Following Muscular Fatigue.
(30 mg histamine-base in Beeswax daily.)

Dog No.	Initial wt, lbs	Days on treadmill	Total hrs on treadmill	No. of hist. inj.	Wt loss lbs	Results
888	39	6	23	6	4	3 gastric ulcers
902	32	6	20.5	6	3	Negative G. I. T.
909	37	5	18	5	3	Duod. ulcer and erosions
916	34	5	18.5	5	2	Multiple duod. and jejunal erosions
948*	40	2	6	1	—	Duod. and jejunal erosions
951	37	5	19	6	3	Large duod. ulcer, antral erosions
956	26	5	19	6	7½	Large duod. ulcer, antral erosions
967	43	5	17	6	6½	2 duod. ulcers
979	45	5	20	5	4	Negative G. I. T.
994	40	5	19	5	2	Duod. erosions
7	32	3	12	3	5	Died, perforated duod. ulcer
16	43½	5	20	5	½	Negative G. I. T.
38	54	5	20	5	4	Duod. erosion
59	44	5	20	5	9	Multiple penetrat. duod. ulcers
375	38	5	20	5	5½	Negative G. I. T.

* Died in hyperthermia (rectal temperature above 108° F).
No. of dogs tested 15
No. of dogs with ulcer and/or erosion 11

in a good general state of nutrition, free of any infection and secreted clear pouch juice. To facilitate the collection of the gastric juice without spillage on the treadmill both pouches were made with a 30 French mushroom catheter as an outlet for the gastric juice. The test period was begun at 9:00 a. m. Routinely food pans were removed from cages at 5:00 p. m. on the day preceding the test insuring a 16-hour fasting period before each experiment. Water was allowed ad lib up to but not after the time of the onset of the experiment. The samples were collected at one-half-hour intervals. The pouches were tested alternately on and off the treadmill. Dogs tested on the treadmill were run for 20 to 30 minutes before the collection of gastric juice was begun. All samples were analyzed the same day for volume, free and total acid, and pH, volume permitting.

In order to test the depressant action of muscular fatigue on gastric secretion an artificially produced stimulation of pouch secretion was created using one-half milligram histamine-base (1.37 mg histamine phosphate) injected subcutaneously. All dogs were given one or more days rest between experiments. Diet consisted of horsemeat, milk, and dog biscuits fed at approximately the same time each day.

Exp. I. *Results.* The incidence of histamine-in-beeswax provoked ulcer or erosion gastric and/or duodenal) following strenuous muscular exercise is shown in Table I. This incidence is to be compared with the results as shown in Table II of injection of the same dose of histamine-in-beeswax for a comparable length of time without the effect of fatigue. Of the 2 dogs which were run on the treadmill as outlined above but were not given histamine-in-beeswax injections neither developed ulcers or erosions. However, at autopsy one of these animals (No. 326) showed a remarkably severe duodenitis. The duodenum was fiery red in color due to submucosal hemorrhages from the pylorus to the point of attachment of the ligament of Treitz. Microscopically sections from this area showed intense dilatation of the capillaries within the mucosa with extravasation of red blood cells. The colon likewise showed a rather severe colitis, the mucosal surface being bright red in color due to congestion and submucosal hemorrhages. This appearance of the colon was quite a common finding at autopsy also in the dogs run on the treadmill and given histamine. (Table I).

The blood changes caused by this amount of exercise on the treadmill were a mild hemoconcentration, a moderate fall in blood sugar immediately following exercise, and a polymorphonuclear leucocytosis above 25,000

TABLE II.
Production of Ulcer in Normal Dogs.
(30 mg histamine-base in Beeswax daily.)
Control Series—No Muscular Fatigue.

Dog No.	Initial wt, lbs	No. of hist. inj.	Wt, loss lbs.	Results
960	40	6	4	Negative gastrointestinal tract
980	31	5	—	" " "
993	35	5	1	Single duodenal erosion
5	37	5	2½	Negative gastrointestinal tract
11	47	5	4	" " "
52	22	5	2½	" " "
65	32	5	4	Duodenal ulcer
380	30	6	4½	Negative gastrointestinal tract
282	23	5	3¾	" " "
870	17	6	0	" " "
996	15	5	0	" " "
12	18	5	2	Single duodenal erosion
51	21	5	1½	Negative gastrointestinal tract

No. of dogs injected 13
No. of dogs with ulcer and/or erosions 3

TABLE III.
Effect of Muscular Fatigue on Gastric Pouch Secretion.
Average Values.*

Dog No.		Fasting Vol., cc	Free acid	1st ½ hr after histamine‡ Vol. cc	1st ½ hr after histamine‡ HCl output mg/½ hr	2nd ½ hr after histamine Vol. cc	2nd ½ hr after histamine HCl output mg/½ hr	3rd ½ hr after histamine Vol. cc	3rd ½ hr after histamine HCl output mg/½ hr
689	Resting	2.26	Absent 4 out of 9 trials	9.22	44.75	5.38	27.97	0.58	qns.
	Fatigue	0.21	Absent 7 out of 10 trials	3.79	16.90	4.13	20.31	0.35	qns.
21	Resting	0.37	Absent 1 out of 6 trials	8.20	37.26	15.43	89.11	3.31	18.55
	Fatigue	0.09	Absent 3 out of 5 trials	7.10	33.69	8.30	47.10	1.26	5.72

* Dog 689 tested 19 times.
Dog 21 tested 11 times.
‡ Dose of histamine phosphate 1.37 mg aqueous solution subcut.

which persists as long as 24 hours after the termination of a 4-hour period on the treadmill.

All dogs on the treadmill showed an elevation of rectal temperature which was roughly correlated with the amount of fatigue and their physical condition. The average elevation was 2°F.

Exp. II. *Results.* Studies of the effects of muscular fatigue on gastric secretion are summarized in Table III. Both dogs show a depression of volume and free acidity of the fasting (basal) pouch secretion. Moreover, the basal pouch secretion was noticeably more mucoid (often jelling in the bottom of the tube) when the animal was fatigued. Upon the background of an artificially introduced stimulus to gastric secretion in the form of an injection of aqueous histamine the depressant effect of muscular fatigue becomes much more apparent upon the volume of gastric juice. After aqueous histamine stimulation in exercised animals there is also a consistent, but small, reduction in degrees of free and total acid in the pouch juice. However, the main depressant effect on the production of total hydrochloric acid in muscular fatigued animals is the reduction in volume of juice secreted. The results in Table III are reported in terms of weight or output of hydrochloric acid per unit time which is the most reliable criterion of the rate of acid secretion in the stomach. (Lim).[2]

Discussion. These results indicate that mod-

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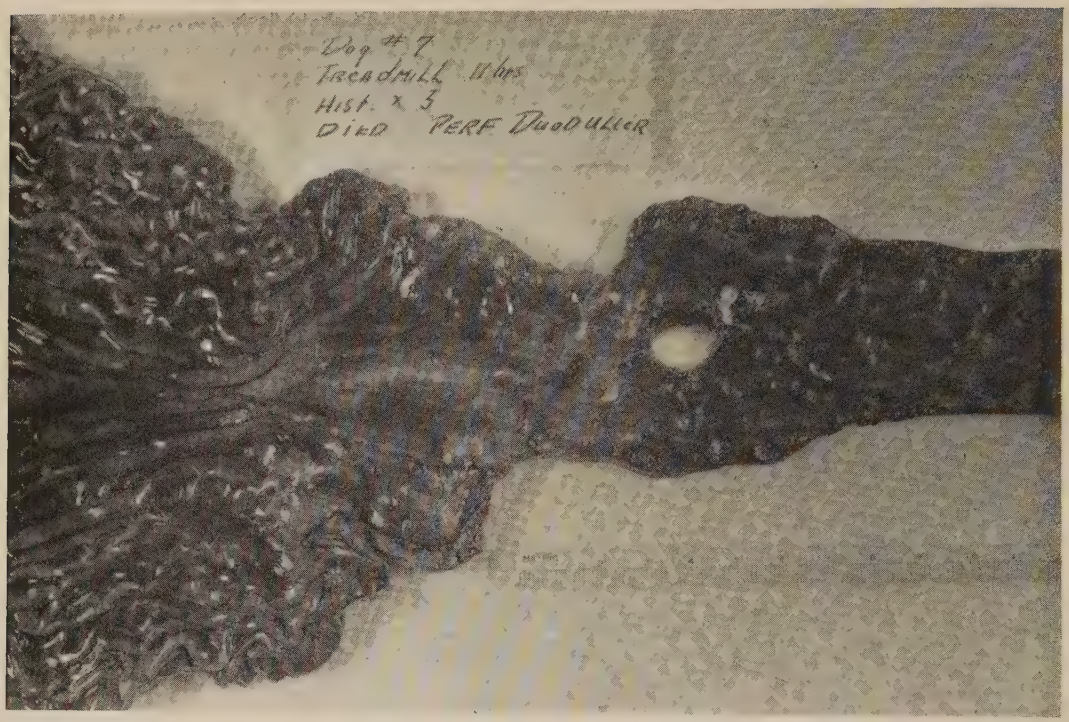

FIG. 1.
Stomach from dog No. 7, dying with a perforated duodenal ulcer after being run a total of eleven hours on the treadmill over a three-day period and at the same time receiving a daily injection of histamine-base.

erately severe muscular fatigue abets the histamine-provoked ulcer quite appreciably while at the same time depressing the output of hydrochloric acid. These seemingly paradoxical findings would suggest that the ulcer abetting effect of muscular fatigue is an effect on the mucosa itself (most probably on its blood supply) which renders it more vulnerable to the digestive action of the acid-pepsin mixture. There seems little reason to doubt that strenuous muscular exercise would cause a disturbance in the blood flow through the gastrointestinal tract by partitioning available blood volume in favor of the skeletal muscles. Evidence from this laboratory has shown that vasoconstriction[3] produced by adrenalin-in-beeswax injection or by liberation of adrenalin due to exposure to cold[4] may aid and abet the ulcer diathesis by creating such anoxic areas that fail to resist the digestive action of gastric juice. Muscular fatigue[5] is likewise known to cause the liberation of adrenalin.

The greater weight loss in the experimental group (Table I) was due in part to the muscular exertion and in part due to the earlier development of an ulcer with consequent anorexia.

The hyperthermia associated with muscular fatigue may be a factor in the suppression of gastric secretion observed.

Conclusions. 1. Muscular fatigue in dogs abets the occurrence of the ulcer produced by chronic histamine stimulation.

2. Muscular fatigue in the dog depresses the output of hydrochloric acid in gastric juice from Heidenhain pouches both under basal conditions and after an artificial stimulation (aqueous histamine) due principally to a reduction in the volume of juice secreted.

[3] Baronofsky, I., and Wangensteen, O. H., *Bull. Am. Coll. Surg.*, 1945, **30**, 59.

[4] Lillehei, C. W., unpublished data.

[5] Gellhorn, E., *Autonomic Nervous Regulations*, Interscience Publishers, Inc., New York, N. Y., 1943, p. 135.

16201

Attempts to Prevent Ergot Gangrene with Heparin and Dicumarol. Vascular Effects of Ergot by Fluorescein Technic.

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The suggestion that thrombosis, associated with ergot gangrene,[1–10] contributes to the vascular occlusion responsible for the resultant tissue necrosis,[3,7] prompts the hypothesis that prevention of thrombosis should modify the gangrene. This hypothesis is supported by the observation that heparin prevents necrosis resulting from experimental "frostbite," a condition with which thrombosis is also associated.[11–13]

The present study is concerned with the influence of heparin and dicumarol on the incidence of ergotamine induced gangrene in the rat.

Gangrene of the tail was produced in male and female albino rats (140-200 g) by the subcutaneous or intraperitoneal injection of a 1:1000 solution of ergotamine tartrate. Whenever a particular treatment was employed, litter mates served as controls. Preliminary studies revealed no influence of sex or route of administration of ergotamine on the incidence of gangrene.

Gangrene can be produced by a single injection of a suitable dose of ergotamine[6,14–17] and such a procedure was employed. When doses of 3.3, 12.5, 25.0, 37.5 and 50.0 mg/kg were injected it was observed that the incidence of gangrene with the smallest dose was very low, while with the largest dose a high mortality occurred. The incidences of actual necrosis in 31, 17 and 13 normal rats given 12.5, 25.0 and 37.5 mg/kg of ergotamine tartrate were 35.5, 43.3, and 15.4% respectively.

In the process of developing gangrene the appearance of the rats' tails passed progressively through several poorly defined stages. The earliest certain indication of impending necrosis was the appearance of a demarcating region of redness and tenderness, distal to which was an area of extreme pallor, with dark discoloration of the tip of the tail. This change was designated arbitrarily as the onset of gangrene, and on the basis of such criteria the time of onset of gangrene was determined in 115 rats (Fig. 1). A few animals began to develop gangrene within 24 hours of the injection of ergotamine. Approximately 50% of the animals which developed gangrene began to do so within 3 days and 100% within 7 days of the injection of ergotamine.

Rats given a single subcutaneous injection of 30 mg/kg of heparin in Pitkin's menstruum[18,19] showed prolongation of the blood

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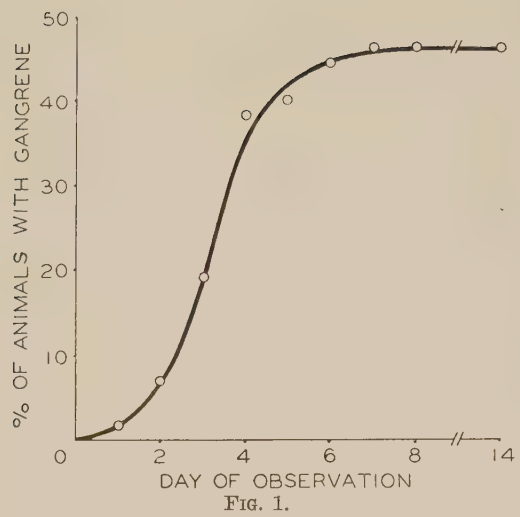

FIG. 1.

clotting time. This effect was maximum at 24 hours, and persisted for 36 hours. Clotting times were estimated by the capillary tube method on blood obtained from the tail. Following 3 daily doses of 30 mg of heparin in Pitkin's menstruum, the average clotting time exceeded 20 min. (normal = 1.04 ± 0.38 min.).

In view of the evidence that 50% of the control animals began to develop gangrene within 3 days of the injection of ergotamine, it was reasoned that therapy with anticoagulants need not be continued beyond this time. Thus, in the present series of animals, 5 daily doses of 30 mg/kg of heparin in Pitkin's menstruum were administered, beginning 2 days prior to the injection of ergotamine.

The incidences of actual necrosis in the series of 10, 8 and 5 heparin-treated rats given 12.5, 25.0 and 37.5 mg/kg of ergotamine tartrate were 30.0, 50.0 and 40.0% respectively, and all animals which developed gangrene did so within 6 days of the injection of ergotamine.

Rats given 2.5 mg of dicumarol by mouth show changes in prothrombin times which are maximum at 24 hours and last 36 hours. These animals will tolerate 10 mg of dicumarol daily for 3 days.[20]

[20] Overman, R. S., Stohman, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1942, **142**, 941.

Preliminary to the present study a series of rats were given 5 mg of dicumarol daily for 10 doses. Clotting times determined during this course of therapy were never above 8 min. However, bleeding from the tip of the tail after collection of blood for clotting determinations was greatly prolonged and 2 of 7 animals bled to death from this minor wound. Some animals developed spontaneous hemorrhages which stopped when dicumarol was discontinued and some showed severe anemia associated with tarry stools. The daily administration of 5 mg of dicumarol thus appeared to represent an effective and near maximum tolerated dose in the rat. In the present series 5 such daily doses were administered, beginning 2 days prior to the injection of ergotamine.

The incidences of actual necrosis in the series of 13, 16 and 6 dicumarol-treated rats given 12.5, 25.0 and 37.5 mg/kg of ergotamine tartrate were 15.4, 31.3 and 100% respectively, and all animals which developed gangrene did so within 6 days of the injection of ergotamine.

Statistical analysis of the pooled data from the control, heparin and dicumarol experiments for the incidence of gangrene, as a function of the dose of ergotamine, revealed that no significant difference in the incidence of gangrene was attributable to the dose of ergotamine administered. Therefore, in evaluating the incidence of gangrene as a function of the treatment, the animals in the 3 dosage groups under any particular regime were pooled to give a figure representing the incidence for the group (Table I).

The incidences of gangrene in the control, heparin treated and dicumarol treated groups were 35.1, 39.1 and 37.1% respectively. Statistical analysis revealed no significant difference between these groups.

There would appear to be two possible explanations for the failure of heparin or dicumarol to reduce the incidence of ergotamine induced gangrene in the rat. They are (1) thrombosis does not contribute to the development of gangrene or (2) the anticoagulants employed were ineffective in preventing thrombosis.

Microscopic examination of the tails of

TABLE I.
Influence of Anticoagulants on Incidence of Ergotamine-Induced Gangrene in Rats.

Procedure	Total	Mortality No.	Mortality %	Survivors	Gangrene No.	Gangrene %
Control	82	8	9.8	74	26	35.1
Heparin	28	5	17.9	23	9	39.1
Dicumarol	38	3	7.9	35	13	37.1
X^2*		1.19			0.012	
p†		>0.50			>0.99	
Significance		None			None	

* Corrected Chi Square.
† Probability.

TABLE II.
Usefulness of Fluorescin Test in Predicting the Development of Ergot Gangrene in Rats.

	Ultimate incidence of gangrene					
	Group I (Tested 6 hr after ergotamine injection)			Group II (Tested 24 hr after ergotamine injection)		
		Gangrene			Gangrene	
Results of test*	Total	No.	%	Total	No.	%
Fluorescence	11	4	34.9	18	5	27.8
No fluorescence	66	29	44.0	19	15	79.0
X^2†		0.019			7.79	
p‡		0.90			<0.01	
Significance		None			High	
Ass'n Coef.§		—			0.417	

* Presence or absence of fluorescence of tail 1 hr after injection of 1 cc 10% fluorescein.
† Corrected Chi Square.
‡ Probability.

$$\S = \sqrt{\frac{X^2}{n + X^2}}$$

2 rats, in which gangrene was developing in spite of therapy with dicumarol, revealed that the vessels were largely free of blood and there was no evidence of thrombosis. Similar studies on 2 control animals, developing gangrene, revealed accumulated masses of red cells containing small amounts of fibrin in the large arteries, but no actual thrombosis was observed. It is thus concluded that thrombosis is incidental, rather than contributory, to the vascular occlusion resulting in ergot gangrene and the severity of the vascular impairment is to be explained solely on the basis of the vasoconstriction produced by the ergot alkaloids.

In order to gain further information on the severity of the vascular impairment which can be produced by such vasoconstriction, one cc of 10% sodium fluorescein was injected intraperitoneally into 12 normal and 23 ergotamine-treated rats. Fluorescence appeared in the ears of normal rats 2.7 ± 0.9 min. and in the tails 9.5 ± 1.91 min. after the injection of the dye. In the animals receiving ergotamine (12.5 or 25.0 mg/kg) the time required for the appearance of fluorescence in the ear was 9.32 ± 4.06 min. while in the tail 244 ± 182 min. was required. It is thus concluded that the circulation to the tail of rats receiving ergotamine is not only greatly, but preferentially impaired.

This preferential impairment of the circulation to the tail might be due either to the degree or the extent of the vasoconstriction occurring in this structure. In a series of ergotamine treated animals (37.5 mg/kg) no

bleeding occurred when the tail was amputated within 1-2 cm of the base. Normal animals, so amputated, showed vigorous hemorrhage and even spurting of blood. The failure of the major arteries of the tail to bleed when transected implies practically complete closure of these vessels by ergotamine.

The time required for fluorescence to appear in the tail of rats receiving ergotamine is not to be construed as a measure of the duration of the action of ergotamine on this vascular bed. A highly diffusable substance, such as fluorescein, could slowly accumulate to a sufficient concentration to give fluorescence of the tissues, even in the presence of severe vasoconstriction. A different type of test was devised to give information as to the duration of the vascular impairment produced by ergotamine.

Seventy-seven rats were given fluorescein within 6 hours after having received ergotamine and another group of 37 rats were given the dye 24 hours after ergotamine. It was arbitrarily decided that all animals in which the time required for fluorescence to appear in the tail was longer than one hour should be classed as having a seriously impaired circulation. In the group tested within 6 hours after the administration of ergotamine 66 or 85.7% of the animals had seriously impaired circulation to the tail. In the group tested 24 hours after the administration of ergotamine 19 or 51.1% had seriously impaired circulation to the tail. Thus, in approximately half of the animals a single dose of 12.5-25.0 mg/kg of ergotamine can cause nearly complete vascular occlusion of the tail vessels, lasting for at least 24 hours.

It can be seen in Table II that a significantly greater incidence of gangrene developed in those animals which, by the fluorescein test, still showed seriously impaired circulation to the tail 24 hours after the administration of ergotamine, than in those which did not. The coefficient of association between the performance on the fluorescein test carried out at 24 hours and the incidence of gangrene is of such magnitude (0.417) as to imply that the test is of considerable value in estimating the prognosis.

It may be assumed that an agent, to be effective against such gangrene, should be able to bring about fluorescence in a tail in which it did not appear as a consequence of the action of ergotamine. Priscol, papaverine, magnesium sulfate, aminophylline, nicotinic acid, alcohol, ether, demerol, histamine, methacholine and histidine-ascorbic acid were shown to be incapable of producing such an effect, and the tails of animals so treated remained nonfluorescent.

Summary. Twenty-four hours after the administration of a single dose of ergotamine tartrate, approximately 50% of rats showed, by means of the fluorescein test, a seriously impaired circulation to the tail, due to marked constriction of the major arteries of the tail. Forty to 50% of rats receiving a single dose of 12.5-37.5 mg/kg of ergotamine tartrate may be expected to develop gangrene of the tail and to begin to do so within one week of the injection of ergotamine. Anticoagulants, such as heparin and dicumarol, do not alter the incidence of ergotamine induced gangrene. It is concluded that the thrombosis, observed to be associated with this process by others, is incidental rather than contributory to the vascular occlusion.

16202

Effect of Intrasplenic Injections of Alloxan in the Rat.*

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Alloxan is usually effective in producing diabetes in experimental animals whether it is administered subcutaneously or intramuscularly,[1] intraperitoneally,[2] intravenously[3] or enterally.[4] This drug has a greater diabetogenic action when given to rats which have been fasted 2 or 3 days.[5] Furthermore, the selective action of alloxan, in destroying certain cells of the pancreatic islets, takes place within a few minutes following its administration.[6,7]

Since the action of alloxan is limited to such a short period of time following its administration, the present study has been undertaken in order to determine whether the liver might be responsible for the inactivation of this substance. Intrasplenic injections permit the alloxan to pass through the liver before it reaches the pancreas.

Material and Methods. One hundred and twelve adult male rats of the Long-Evans strain were used in this study. The animals were given intrasplenic injections of a 25% solution of alloxan and the dosages used were 50, 88, 125 or 150 mg/kg. The rate of injection, controlled by a micrometer syringe, varied in the different animals but the most satisfactory rate tried was 1/100 cc/min. Control rats received either intraperitoneal or subcutaneous injections of alloxan in the same amounts and at the same injection rate as was injected intrasplenically.

The rats were placed in metabolism cages for urine collections on the eighth and fifteenth days, respectively, following the injection of alloxan. Only rats with a 2-4+ urine sugar were considered to be diabetic. At the end of the 15-day period samples of tissue were taken for histological examination. Both the splenic and duodenal portions of the pancreas and pieces of liver were fixed in Bouin's solution.

Results. Considerable variation was seen in the response of the rats to the intrasplenic as well as the intraperitoneal injections of alloxan. Certain animals were not able to tolerate the toxic effects of the drug, regardless of the route of administration and differences in the injection rate and the amount of alloxan administered accounted for some variation. The data for the animals which were not fasted before they received the alloxan are shown in Table IA. The rate at which the injections were given is not indicated on the table. In most instances, however, the control rats received intraperitoneal injections of the same amount of alloxan, at the same rate, as did the animals which were given injections intrasplenically. The smaller amounts of alloxan were usually more effective in producing diabetes when injected rapidly into the spleen than when given intraperitoneally. However, if 125 mg/kg were injected rapidly into the spleen, the animals usually died. Death in such cases was probably due, in many instances, to infarcts in the liver. The lobes of such livers showed a marked necrosis, although the crown region was generally normal.

Animals which received the drug intrasplenically tolerated it much better when injections were made at a slower rate (1/100 cc/min.) but a given amount of alloxan was

* This study was aided by a grant from Hoffmann-LaRoche, Inc.

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TABLE I.
Effect of Alloxan Injections.

	Mg alloxan/kg body weight						
	A Non-fasted				B Fasted 2 days		
	50	88	125	150	88	125	150
A. Intrasplenic							
1. Diabetic	1	2	4			7	2
2. Non-diabetic	8	1	1	3	3	1	
3. Died	1	5	16	1			
B. Intraperitoneal							
1. Diabetic			7			6	2
2. Non-diabetic	4	4	6	2	3	4	
3. Died	2	3	3				
C. Subcutaneous							
1. Diabetic						8	
2. Non-diabetic						4	

less effective in producing diabetes.

A more uniform response was obtained and no deaths occurred when the rats were fasted 2 days before they were injected slowly with alloxan (Table IB). In the rats studied, 88 mg of alloxan per kg was not sufficient to elicit diabetes when given intrasplenically or intraperitoneally, while 150 mg resulted in a severe diabetes when injected by either method. When 125 mg/kg was given, diabetes was produced in 7 out of 8 rats when injected intrasplenically, in 8 out of 12 rats when injected subcutaneously, and in 6 out of 10 rats when given intraperitoneally. Within the limitation of the number of animals used in this study it was noted that diabetes in the latter group of animals was not so severe as in the other 2 groups.

The islets in both the splenic and duodenal portions of the pancreas were examined histologically in an attempt to determine whether one portion of this gland might be more susceptible to the alloxan. Islet damage, however, was not localized in any particular region of the pancreas.

Comment. Since alloxan injected intrasplenically is just as diabetogenic as when given subcutaneously, indications are that this drug is not inactivated specifically in the liver. However, when administered intrasplenically, alloxan appears to be slightly more effective in producing diabetes than when given intraperitoneally. A possible explanation for this variation is that after the latter method of administration, the alloxan is absorbed over a large area of the peritoneal cavity and is thus diluted to a greater extent before reaching the pancreas.

Summary. The present observations indicate that alloxan is just as effective as a diabetogenic agent when given intrasplenically as when given subcutaneously. Similar amounts of alloxan given intraperitoneally result in many instances in a milder diabetes. It appears that alloxan is not inactivated specifically in the liver.

16203

Effect of Fagarine on Auricular Fibrillation.*

David Scherf.

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In 1932 it was demonstrated that an alkaloid from an Argentinian plant, *Fagara coco*, exerts a depressant action on the heart.[1] Subsequent investigations showed that the purified alkaloid, fagarine, raised the fibrillation threshold during faradization of the ventricles[2] and that it may prevent the fibrillation which follows ligation of the coronary arteries.[3]

The action of fagarine on the established auricular fibrillation has not been tested because no method was available to elicit this disturbance for a period of sufficient duration without employing other measures which would influence the results. Post-faradization fibrillation is not stable and changes, as a rule, quickly into normal sinus rhythm. Auricular fibrillation persists for a longer time if faradization is combined with the administration of physostigmine, muscarine or choline derivatives. These substances, however, have side-effects on the cardiac muscle which make an evaluation of the experimental results difficult. If, however, 0.05 cc of a 0.05% solution prepared from aconitine crystals is injected subepicardially into the area of the sinus node in dogs it leads to auricular fibrillation or auricular flutter of long duration and permits the study of the effect of rapidly acting drugs. Such attacks of fibrillation occasionally last for more than an hour.[4]

Method. In 10 dogs the chest and the pericardium were opened in the usual way under nembutal anesthesia and artificial respiration. Both vagi were severed in the neck in 7 experiments. The electrocardiogram was taken in Lead II. After the appearance of persistent auricular flutter or fibrillation 0.004 g/kg of alpha-fagarine chloride was injected intravenously.

Results. In all 10 experiments the flutter or fibrillation disappeared within one minute after the injection ended. Fig. 1a shows auricular fibrillation and its sudden change into sinus rhythm with a rate of 250 following an injection of 0.03 g of fagarine. In Fig. 1b auricular impure flutter changes into a sinus rhythm with a rate of 166. The sinus tachycardia following the disappearance of fibrillation is due at least in part, to the division of the vagi. In those experiments in which the vagi were intact the results were the same.

Table I gives the time intervals between the end of the intravenous injection of fagarine and the disappearance of the prevailing arrhythmia.

Following the injection of fagarine, faradic stimulation of the vagi, even with strong currents, had only a very slight effect on the cardiac rate and on a-v conduction. If larger doses of fagarine are used (0.01/kg) the faradic stimulation of the vagi is without effect.[2]

Inspection of the exposed heart showed no evidence of dilatation or any disturbance of contractility following the fagarine injection. This should be emphasized because definite dilatation and weakening of cardiac contractions does appear immediately if quinine or quinidine is injected in a dose sufficient to abolish fibrillation. A slight prolongation of the a-v conduction time, amounting to only 0.01-0.02 second appeared at the height of fagarine effect, and the RS-T segments were slightly depressed but no other changes were found in the electrocardiogram. This point

* Our thanks are due to the Laboratories Apotarg in Cordoba, Argentina, for the supply of the fagarine. This study was supported by a grant from Bernhard Altmann.

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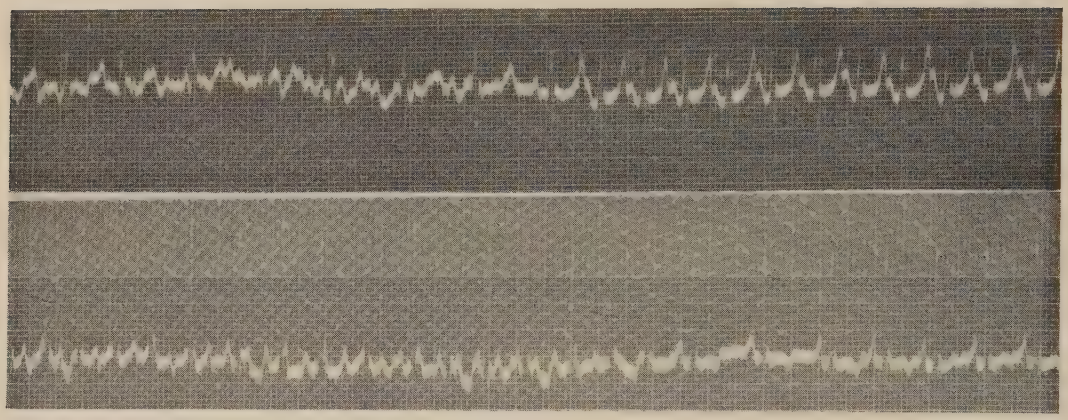

FIG. 1.
Fig. 1a (upper figure) shows the termination of auricular fibrillation and Fig. 1b (lower) the termination of auricular impure flutter by an intravenous injection of fagarine.

TABLE I.
Time of Disappearance of Arrhythmia.

Date	Prevailing arrhythmia	Time of disappearance of arrhythmia, seconds	Remarks
9/30	fibrillation	48	vagi intact
10/7	"	36	" severed
14	flutter	55	" intact
21	fibrillation	12	" severed
11/11	"	25	" "
25	"	18	" intact
1/7	"	25	" severed
2/26	"	22	" "
4/22	flutter	31	" "
5/13	fibrillation	42	" "

is also emphasized because the clinical administration of the drug occasionally leads to serious disturbances of stimulus formation in the ventricle.

The systolic blood pressure showed a temporary rise lasting only 2 to 5 minutes and amounting to 30-40 mm Hg. In all experiments the original values of blood pressure were registered within 5 minutes.

Following the injection of fagarine renewed subepicardial injection of the aconitine solution had no effect on the existing sinus rhythm for about 45 minutes. After this time it was possible to elicit tachycardias and fibrillation for a second time with this method.

Summary. The intravenous injection of fagarine in dogs with an auricular flutter or fibrillation caused by aconitine abolishes these arrhythmias within one minute and leads to the reappearance of sinus rhythm.

16204

Enzyme Studies on the "Endocrine Kidney."

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Selye[1] reported a technic for the transformation of the kidney into a purely endocrine organ in the rat. This technic consists in partially constricting the aorta between the origins of the 2 renal arteries by means of a ligature adjusted so that the blood pressure in the kidney below the constriction (in the rat the renal arteries leave the aorta at different levels) decreases to a point where no filtration occurs. To further insure against filtration taking place in that kidney the ureter is tied off and cut. The circulation of blood remains adequate, however, for the nutrition of the organ. Rats so treated have been shown to develop a very acute form of malignant hypertension.[2] The present experiment deals with the determination of the arginase and phosphatase content of these kidneys, which are non-functional as regards urine formation.

Procedure. Twelve female albino rats weighing between 150 to 160 g were used in our experiment. The operation as described by Selye and Stone[2] was performed under ether anesthesia. Seven days later the animals were sacrificed and the kidneys removed, weighed and placed in separate vials containing 5 ml of distilled water; the right (normal) kidneys served as controls. Five samples were then sent fresh by air mail to Rochester where they were analyzed for the various enzyme activities by the previously described procedures.[3-6]

Results. There is a marked, indeed almost a complete, disappearance of both arginase and "alkaline" phosphatase from the "endocrine" kidney (Table I). The "alkaline" phosphatase was determined also with the addition of magnesium sulfate[7] to the substrate mixture in order to rule out the possibility of lack of activator instead of a decrease in amount of this enzyme. The increase in activity was identical for the 2 sets of tissues, 47.7 and 46.7% for the "endocrine" and normal kidneys respectively. The change in "alkaline" phosphatase is in agreement with the observations by Wilmer[8] on the kidneys of rabbits and rats after ligature of the ureters.

TABLE I.

		Arginase		Phosphatases			
				"Alkaline"		"Acid"	
Kidney	Wt, mg	Total U	U/g	Total U	U/g	Total U	U/g
Normal	811	64	99	109	141	15.0	18.2
Endocrine	357	6	17	5.5	16	6.0	16.6
% diff.	—56	—91	—83	—95	—89	—60	—8.8

U = Units.

* Fellow of the Canadian National Research Council.

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It is of special interest that the "acid" phosphatase, in contrast to the other enzymes, decreased in total amount but not in concentration (units per gram of tissue). It would seem, therefore, that this enzyme is necessary for the integrity of the cell and perhaps its endocrine function while the "alkaline" phosphatase and arginase are necessary only in connection with urine formation.

Summary. In "endocrine kidneys" there is a marked indeed almost a complete disappearance of both arginase and "alkaline" phosphatase. On the other hand the "acid" phosphatase decreases in total amount but not in concentration.

16205

Activation of Hypertensin and Tyrosine by Subthreshold Amounts of Epinephrine.*

E. MYLON AND J. H. HELLER. (Introduced by M. C. Winternitz.)

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Fractions of hog kidney containing renin do not produce constriction of the vessels of the rabbit's ear until subthreshold amounts of epinephrine are added to the perfusate.[1] Similar vaso-activation of liver fractions by epinephrine has been noted when they are combined with subthreshold amounts of epinephrine.[2] Fresh plasma is also capable of activating liver fractions.[3] This last observation is of interest because of the report that vaso-inactive renin-containing kidney fractions become vasoconstrictive when plasma is added to the ear perfusate.[4] This was ascribed to the formation of hypertensin rather than to the activation of renin by plasma. Since a similar constriction occurs following the addition of plasma to liver fractions under circumstances that would seem to be incompatible with the formation of hypertensin, a reinvestigation of the action of hypertensin on the vessels of the rabbit's ear was undertaken.

The experiments that follow show that hypertensin, a protein fraction, constricts the vessels of the rabbit's ear only when the perfusate is supplemented with traces of epinephrine or fresh plasma. This suggested the possibility that parts of the protein molecules, *e. g.,* peptides or even amino-acids, are responsible for the observed effect. Accordingly, the studies were extended to encompass a large majority of the known amino-acids. Special attention was directed to tyrosine, tyrosine-containing peptides, and a tyrosinamide in accord with Cruz-Coke's[5] hypothesis that tyrosine molecules are necessary for the *in vivo* action of hypertensin.

Materials and Methods. Hypertensin was prepared from purified hog renin with the bovine globulin Fraction IV-1 (Armour) serving as substrate. Three hundred cc of a 1% solution of substrate were buffered at pH 7.5 and incubated for 10 minutes with 2-3 Swingle units of renin. N1 HCl was then added to bring the pH to 5.2 and the mixture immersed in boiling water for 10 minutes. The coagulated proteins were then removed by filtration and the almost water-clear filtrate lyophilized. The lyophilized material was taken up in 16 cc of distilled water and the small residuum of undissolved particles was

* Aided by a grant from the Commonwealth Fund.

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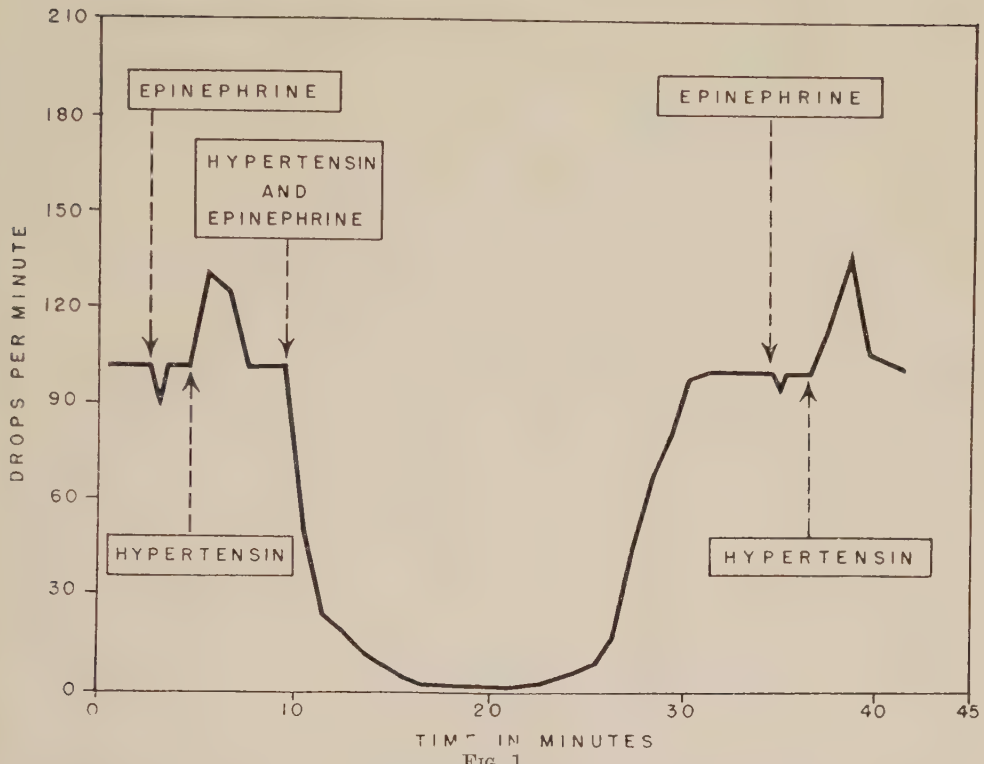

FIG. 1.

While the effect of 1 cc of epinephrine 1:5,000,000 is minimal and the effect of 1 unit of hypertensin is slightly dilating, combination of the two causes a strong and sustained constriction of the vessel of the rabbit's ear.

removed by centrifugation. Four cc of each lot was found to contain between 6 and 8 dog units of hypertensin as tested in the living animal. The remaining 12 cc of each lot were used in perfusion experiments, in amounts equivalent to from one-third to 2 dog units. The ear perfusion technic has been described.[1]

Ears of young albino rabbits seemed to be more sensitive than those of older rabbits and of other types. A preparation was judged to be suitable when the initial drop rate per minute was between 100 and 160, and in addition when an injection of 1 cc of a 1:5,000,000 or 1 cc of a 1:10,000,000 solution of epinephrine caused minor and transient constrictions. Experiments were considered valid only when a terminal injection of epinephrine reproduced a reaction similar to the initial one. The order of the injections with the different materials was varied.

The plasma was prepared according to the technic of Landis.[6] The epinephrine used was Parke Davis 1:1000, and the dilutions were prepared as described previously.[1,2]

The amount of an amino-acid, amide or dipeptide used for a single injection was between 1/100 and 1/200 of a millimole; for *l*-tyrosine it was always 1/200 of a millimole. The pH of the Ringer-Locke solution (7.3) was not changed by the addition of the small amounts of the test substances.

The following amino-acids were used: *dl*-phenylalanine, *dl*-alanine, *dl*-serine, *l*-valine, glycine, *l*-cystine, *l*-tryptophan, *dl*-glutamic acid, *l*-aspartic acid, *dl*-lysine, *l*-leucine, *dl*-isoleucine, *dl*-norleucine, *dl*-threonine, *l*-proline, *l*-tyrosine, *l*-hydroxyproline, *l*-histidine, *dl*-arginine and *dl*-methionine.

The tyrosine-containing dipeptides glycyl-*l*-tyrosine and *l*-tyrosyl-glycin, and also the

[6] Landis, E. M., Wood, J. E., Jr., and Guerrant, J. L., *Am. J. Physiol.*, 1943, **139**, 26.

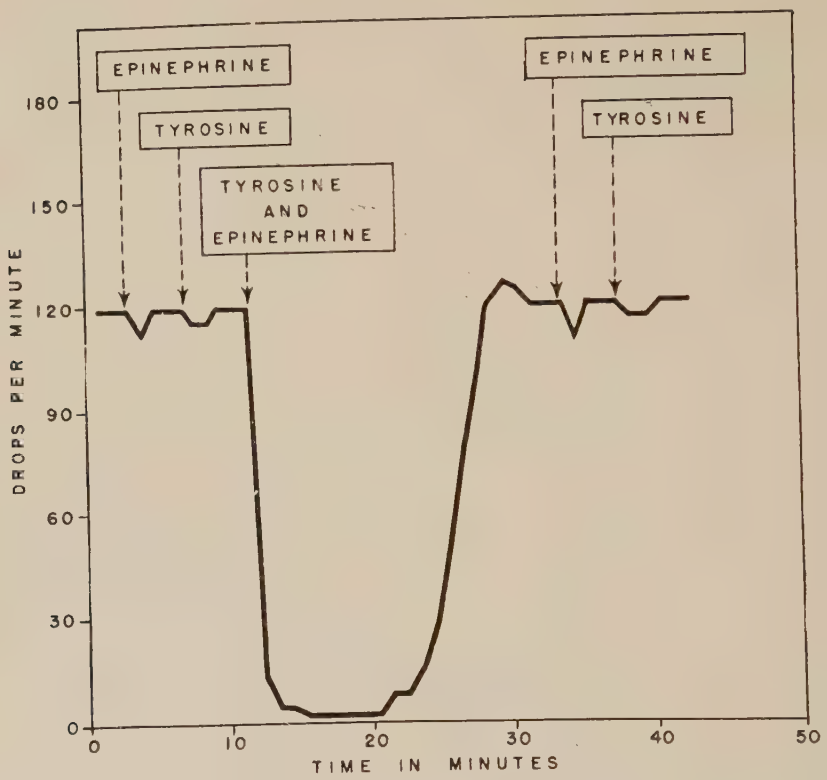

FIG. 2.

Insignificant effect of epinephrine and *l*-tyrosine alone in contrast to the strong vasoconstriction elicited by a combination of both.

Amounts injected:

Epinephrine: 1 cc of a sol. 1:5,000,000.

l-Tyrosine: 0.005 millimole.

tyrosine-amide acetate were tested.†

Iodine was introduced into the tyrosine molecule by slowly adding n/10 aqueous iodine solution to n/1000 solution of *l*-tyrosine until a faint yellow color remained. After keeping the mixture in the dark for several hours, the excess iodine was removed by heating.

Results and Brief Discussion. Hypertensin alone: Neither the initial injection of hypertensin nor its subsequent introductions into the perfusion circuit produced vasoconstriction as determined in numerous experiments with 15 different lots. A slight vasodilation was the usual sequel of the addition of hypertensin to the perfusion fluid.

Hypertensin plus epinephrine: On the other hand, when hypertensin was combined with subthreshold amounts of epinephrine, powerful and sustained vasoconstriction followed. A typical result can be seen in Fig. 1. A total of 28 perfusion experiments were carried out to test the effect of the combination of hypertensin with subthreshold amounts of epinephrine. The vasoconstriction that followed was so intense in 14 instances that the vessels of the ear reduced the drop rate 90 to 99% for 4 to 18 minutes and 100% for 20 minutes. In 10 of the other 14 tests the drop rate was reduced 60 to 90% for from 3 to 8 minutes and in the remaining 4 experiments a more moderate reduction of 50% was observed, lasting from 1 to 8 minutes.

Hypertensin plus plasma: When 1 cc of fresh rabbit plasma was used in place of epinephrine in combination with hypertensin,

† We are indebted to Dr. J. S. Fruton for the generous supply of glycyl-*l*-tyrosine (*l*-tyrosylglycine), and tyrosine-amide-acetate.

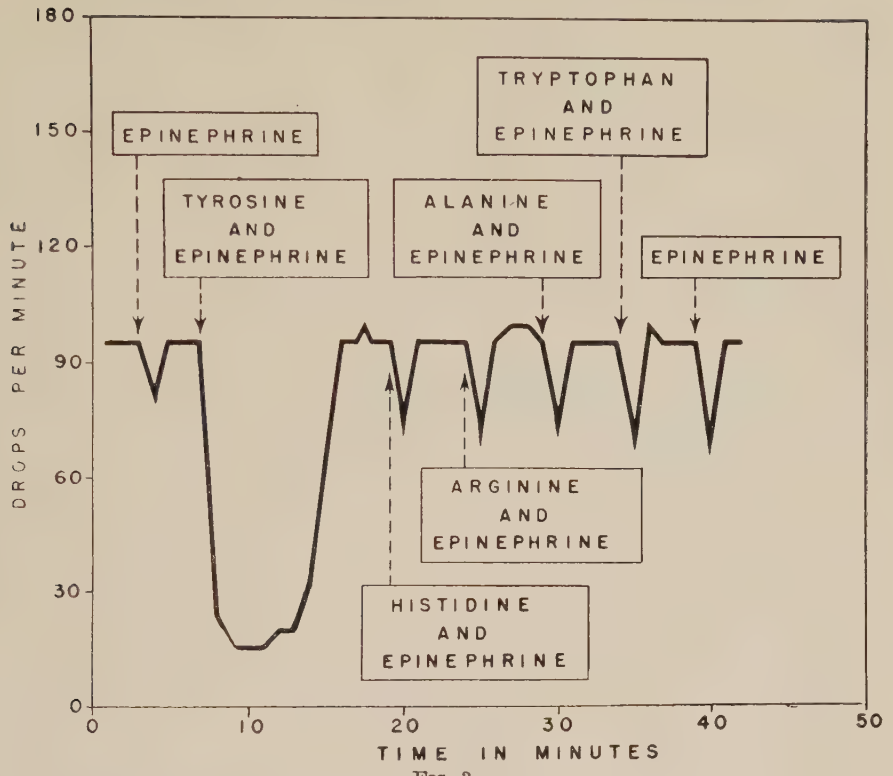

FIG. 3.

Strong vasoconstriction is solely elicited by *l*-tyrosine plus epinephrine and not by a combination of other amino acids and epinephrine.

Amounts injected:

Epinephrine: 1 cc of a sol. 1:5,000,000.
l-Tyrosine: 0.005 millimole.
The other amino acids: 0.01 millimole.

a strong vasoconstriction was elicited. This effect was only obtainable with fresh plasma and incubating the plasma for 8 hours abolished its ability to activate hypertensin. These results are analogous to those previously reported, in which liver fractions were combined with fresh and incubated plasma.[3]

On the basis of the identical results obtained with plasma plus renin[4] and plasma plus hypertensin, it could not be decided whether the observed constriction followed a direct action of plasma on renin or an action of plasma on hypertensin. The specificity of the renin-hypertensinogen reaction on the other hand suggested additional experiments in which it was found that renin itself is being activated. Vasoinactive pig renin, unable to catalyze hypertensin formation with human hypertensinogen, in combination with vasoinactive human plasma produced a significant constriction in the vessels of the rabbit's ear.

Amino-acids: A total of 95 experiments involving one or another of 20 different amino-acids failed to demonstrate any effect when these were added individually to the rabbit's ear perfusate. More important is the fact that with one exception, tyrosine, there was only an inconstant, slight and transient response, for any one compound when traces of epinephrine were added to the perfusate. Addition of epinephrine to the tyrosine-containing perfusate was followed by a sharp and sustained decrease in the drop rate, 45% to 90% for from 3 to 9 minutes (Fig. 2 and 3).

These findings led to the exploration of other tyrosine-containing compounds including glycyl-*l*-tyrosine, *l*-tyrosyl-glycine, and tyrosine-amide-acetate. Needless to say they had no influence when added to the Ringer-Locke perfusate. This status persisted when

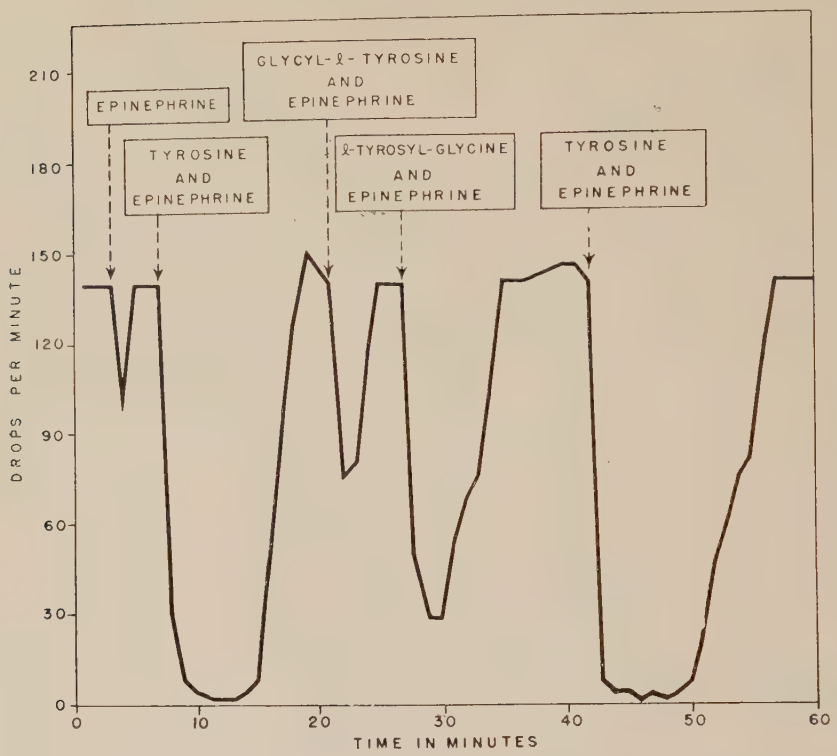

FIG. 4.

The constrictor effect of *l*-tyrosine plus epinephrine is much greater than that of *l*-tyrosyl-glycine plus epinephrine. The effect of glycyl-*l*-tyrosine and epinephrine is insignificant.

Amounts injected:

Epinephrine: 1 cc of a sol. 1:5,000,000.
l-Tyrosine: 0.005 millimole.
The dipeptides: 0.01 millimole.

glycyl-*l*-tyrosine was supplemented with traces of epinephrine. On the other hand, *l*-tyrosyl-glycine and tyrosine-amide-acetate combined with minimal amounts of epinephrine had a moderate constrictive effect on the vessels of the rabbit's ear (Fig. 4).

The vasoconstrictive effect of tyrosine, it would seem, is abolished when the amino group is linked to a carboxyl of a second amino acid. When the amino group remains free and the carboxyl group is bound by another amino-acid or transformed into an amide, the vasoconstrictive effect persists to about 50%.

Another finding still difficult to correlate with the coverage of the amino group of tyrosine-like compounds is the effect of iodinization. This also abolished the vasoconstrictive effect. These results are of interest in relation to Cruz-Coke's suggestions[5] that the pressor action of hypertensin *in vivo* might be related to its tyrosine content.

Summary. Hypertensin has no effect on the vessels of the perfused rabbit's ear. It becomes strongly vasoconstrictive, however, when supplemented with traces of epinephrine and also when fresh plasma is added to the perfusate. None of 20 different amino-acids tested in perfusion experiments on the rabbit's ear had any constrictor effect. When traces of epinephrine were included in the perfusion fluid, tyrosine was the only one of the 20 that exhibited a strong and sustained vasoconstriction. Glycyl-*l*-tyrosine was inactive with and without epinephrine. *l*-tyrosyl-glycine and tyrosine-amide-acetate, both inactive when perfused alone, constricted the vessels if combined with minute amounts of epinephrine. The activity of these two compounds, however, was definitely less than that of tyrosine. Introduction of iodine into the

tyrosine molecule abolished the constrictor action entirely.

The conclusion drawn from these findings is that constriction of the blood vessels of the rabbit's ear is dependent to a large degree upon traces of epinephrine and tyrosine-containing compounds of special configuration.

16206

Prevention of Experimental Dietary Hepatic Cirrhosis by Goitrogenic Substances.

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In previous observations[1] thiouracil, and to some extent thiourea, exerted a beneficial effect in the prevention of dietary cirrhosis of the liver in rats. In animals receiving thiourea the food intake was generally very low and their weights showed a rapidly progressive decline. In contrast, the general well being of rats fed the same basal ration mixed with thiouracil (0.1% in the diet) remained undisturbed. The weight losses sustained were smaller than in the control animals without any appreciable difference in the average food intake in the two groups.

The beneficial effect of thiourea and thiouracil in the prevention of experimental dietary cirrhosis has been related to their effect on thyroid function, specifically to the decreased metabolic rate including the metabolism of protein. Decreased turnover of proteins pertains also to that of their constituents, including methionine, which is one of the key substances in the pathogenesis of dietary hepatic injury.[2]

It is difficult to furnish direct proof for the correctness of this assumption. The demonstration of strict parallelism between goitrogenic activity and preventive effect on hepatic cirrhosis may serve as indirect circumstantial evidence. The superior effect of thiouracil over thiourea is in good accord with these considerations. In this connection it seemed advisable to include propyl-thiouracil and aminothiazole in these investigations. Propyl-thiouracil is at present probably the most effective goitrogenic agent available. Aminothiazole, when first used, was reported to be very effective in the treatment of hyperthyroid conditions.[3] However, pharmacological[4] and more recent clinical studies[5] are at variance with this conclusion and support the view that aminothiazole is less effective and more toxic than propyl-thiouracil or even thiouracil.

Experimental. During the course of our studies extending over several years it became evident that even without any change in the basic composition of the experimental cirrhosis-producing diet the results, such as incidence and severity of cirrhosis, food intake, weight changes, and survival time, may vary at various times. These variations—admittedly within narrow limits—are probably due to changes in the composition of the fat used (perhaps in addition to obscure climatic influ-

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4 Astwood, E. B., Bissell, A., and Hughes, A. M., *Endocrinology*, 1945, **36**, 456.

5 Morgans, M. E., *Lancet*, 1947, **1**, 519; Williams, R. H., *Arch. Int. Med.*, 1947, **80**, 11; McConnell, J. S., Frost, J. W., Wilber, R. W., and Rose, E., *Am. J. Med. Sc.*, in press.

ences), all other experimental conditions being fairly strictly controlled. These observations necessitate the liberal use of controls in experiments in which the effect of a particular substance on the production of dietary hepatic injury is being investigated. In the present report the effect of 6-propyl-thiouracil and of 2-aminothiazole has been studied in two consecutive experiments. In each experiment one group of control animals was placed on the basal experimental ration without goitrogenic substance, to be compared with a second test group of rats receiving the same basal diet supplemented with propyl-thiouracil or aminothiazole. Young adult male rats with an initial weight of 150-225 g were used throughout.

The basal diet consisted of casein (G.B.I.) 8, Crisco 40, sucrose 48, salt mixture 4, with the admixture of 0.05% propyl-thiouracil or 0.05% aminothiazole in the corresponding group of each experiment. Each animal received daily 20 μg thiamine chloride, 25 μg riboflavin, 20 μg pyridoxine, and 100 μg calcium pantothenate, all dissolved in 1 cc of water. Three drops of percomorph oil furnishing 3750 units of vitamin A and 540 units of vitamin D, and 3 mg of mixed tocopherols (Distillation Products) were given weekly. In addition all animals received 50 mg of cystine daily with the purpose of accelerating and aggravating the cirrhotic changes in the liver. As customary, the experimental period was 150 days and those rats that survived were then killed. The final diagnosis of hepatic injury was based, without exception, on microscopic examination. Minor but definite cirrhotic changes were marked with +, whereas more severe cirrhosis was defined as ++ to ++++, with proper consideration for its varying degree in different lobes.

Results. From the tabulation of all the pertinent data collected (Table I, Exp. I) it becomes evident that propyl-thiouracil when added to the basic cirrhosis-producing synthetic diet exerted a marked preventive effect, manifesting itself not only in much lower, almost negligible incidence (8 out of 10 experimental animals were free from cirrhosis) and in milder degree of cirrhosis, but also in

TABLE I.
Effect of Propyl-thiouracil and Aminothiazole on Body Weight, Food Intake, and Hepatic Injury in Rats Fed a Cirrhosis-Producing Diet.

* Exp.	Supplement	No. of animals	Wt loss >50 g	Wt loss <50 g	No wt loss	Avg wt loss, %	Dead before 150 days	Killed at 150 days	Fluid in serous cavities	Food intake, g	Cirrhosis 0	Cirrhosis +	Cirrhosis ++ to ++++	Ceroid 0	Ceroid Trace to +
I.	A. None	12	8	3	1	—29.6 ± 21.7	6	6	5	7.5 ± 0.95	0	1	11	6	6
	B. Propyl-thiouracil	10	0	9	1	—11.0 ± 5.1	0	10	0	3.9 ± 0.23	8	1	1	10	0
II.	A. None	15	3	3	9	— 8.4 ± 26.0	4	11	10	5.8 ± 0.93	1	1	13	10	5
	B. Aminothiazole	15	2	8	5	— 7.1 ± 20.5	2	13	2	6.0 ± 0.62	4	2	9	5	10

absence of serous (partly chylous) effusions in the peritoneal, pleural, and pericardial cavities, in far better survival rate and in more satisfactory weight curves. It was especially impressive to the observer that these better weight curves were obtained with a food consumption only 52% of that of the control animals fed the same diet without propyl-thiouracil. The animals receiving propyl-thiouracil exhibited a perfect fur and were altogether in an exceptionally good general condition.

As would be expected, in the absence of cod liver oil[2,6] and in the presence of hydrogenated fat (Crisco),[7] ceroid was found in the livers even of the control animals in only limited quantity or not at all. Even these irregularly occurring traces of ceroid were missing in the animals fed the diet containing additional propyl-thiouracil. In contrast, the fat content of the liver judged on the basis of histologic examination and not by the more reliable chemical analytical data appeared to stay elevated in both the control animals and in the experimental animals receiving propyl-thiouracil.

The incidence of acute or subacute necrotizing nephrosis[2] happened to have been low in the present group of control animals, only 2 rats out of 12 showing these changes. In the light of this low incidence in the control group, the fact that no renal changes were observed in the group of rats receiving propyl-thiouracil is of interest but of no statistical significance. Atrophy of the testes with suppressed spermatogenesis was seen in 7 out of 10 rats in the control group (A) and in 8 out of 10 in the corresponding group (B) fed propyl-thiouracil. Thus, the injurious effect of the diet on the gonads remained uncorrected by propyl-thiouracil.

In contrast to the remarkable preventive effect of propyl-thiouracil as documented by the data of Exp. I (Table I), aminothiazole in the dose given (0.05%) seemed to influence only very slightly, if at all, the production of dietary hepatic injury in rats (Exp. II, Table I). The slight reduction in the incidence of cirrhosis in the group of rats fed aminothiazole might indicate some beneficial effect but it is of no statistical significance. The only significant change brought about by aminothiazole was with regard to the occurrence of effusions in the various serous cavities: instead of 10 animals out of 15 in the control group (A), only 2 out of 15 in the experimental group (B) exhibited such pathologic manifestations.

Again, in contradistinction to the effect of propyl-thiouracil, aminothiazole exerted no significant influence on ceroid production or on food intake, survival rate, and weight changes of the experimental animals. The liver fat estimated histologically appeared to be high in both the experimental (B) and control animals (A). There was no difference in the incidence of renal or testicular changes between the groups not receiving and those receiving aminothiazole.

Discussion. The present and previous[1] observations are in good agreement regarding the beneficial effect of goitrogenic substances on the prevention of dietary cirrhosis of the liver in rats. The parallelism between goitrogenic and "anti-cirrhotic" potency is satisfactory. Propyl-thiouracil which is considered the most powerful goitrogenic substance proved also to be superior to the other goitrogenic substances tested in its effect on the prevention of dietary cirrhosis of the liver. As regards their relative effectiveness against cirrhosis these substances are in the following order: Propyl-thiouracil > Thiouracil > Thiourea > Aminothiazole. Of course this order applies only to the quantitative conditions chosen for the present and previous experiments. Nevertheless it is of interest to note that on the basis of food intake and molecular weight propyl-thiouracil appears to be at least 5 times as potent in its effect on the prevention of hepatic cirrhosis as thiouracil. The daily dose of propyl-thiouracil amounted on the average to 1.95 mg, which in itself is good support for the assumption that propyl-thiouracil and other goitrogenic substances act with greatest probability through the intermediary of the thyroid gland. So-called lipo-

[6] Endicott, K. M., *Arch. Path.*, 1944, **37**, 49; Endicott, K. M., Daft, F. S., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 330; Wachstein, M., *ibidem*, 1945, **59**, 73.

[7] György, P., unpublished observations.

tropic substances, such as methionine or choline, used for the prevention of hepatic cirrhosis, require relatively higher doses to be effective.

Aminothiazole was found to be without any significant benefit in doses which on the basis of food intake and molecular weight were about 3.5 times higher than those used for propyl-thiouracil.

Successful prophylaxis of hepatic cirrhosis with goitrogenic substances such as thiouracil in the previous[1] and with propyl-thiouracil in the present studies does not necessarily lead to the conclusion that the same approach may be successful in the therapy of this condition. As a matter of fact, in unpublished experiments thiouracil appeared to exert no effect or even a slightly deleterious one on a *pre-existing* cirrhosis in rats.

As an unexpected indirect result of our studies the excellent general nutritional state of the experimental animals receiving propyl-thiouracil deserves special attention. This observation supports the view expressed in another connection by several authors[8] that goitrogenic substances could play an important role in the feeding and fattening of farm animals, and especially in the feeding efficiency, an all-important economic factor. Older experiments were carried out mainly with thiouracil. Our own studies seem to give propyl-thiouracil a definite edge over thiouracil. The practical problem as to whether goitrogenic substances are stored and, if so, how long in animals receiving them with their feed, and whether the ingestion of such animal products may exert a toxic (goitrogenic) effect on the consumer is beyond the scope of this presentation.

Summary. Admixture of propyl-thiouracil (.05%) with a cirrhosis-producing synthetic diet will prevent effectively the production of dietary cirrhosis in rats. This result is achieved with simultaneously reduced food intake and is manifested not only in the absence of cirrhotic changes in the liver but also in the improved general condition of the experimental animals and increased feeding efficiency.

Aminothiazole in the dose used (3½ times the equivalent of propyl-thiouracil) was without any significant effect on the production of dietary cirrhosis, on food intake, survival rate and weight changes.

For the goitrogenic substances tested efficiency in prevention of cirrhosis seems to parallel goitrogenic potency.

8 Kempster, H. L., and Turner, C. W., *Poultry Sc.*, 1945, **24**, 94; Andrews, F. N., and Schnetzler, E. E., *ibid.*, 1946, **25**, 124; Glazener, E. W., and Jull, M. A., *ibid.*, 1946, **25**, 236; Mixner, J. P., Tower, B. A., and Upp, C. W., *ibid.*, 1946, **25**, 536; Reineke, E. P., Davidson, J. A., Wolterink, L. F., and Barrett, F. N., *ibid.*, 1946, **26**, 410; Andrews, F. N., Beeson, W. M., Herrick, E. R., and Harper, C., *J. Animal Sc.*, 1947, **6**, 3; Beeson, W. M., Andrews, F. N., and Brown, P. T., *ibid.*, 1947, **6**, 16; Muhrer, M. E., and Hogan, A. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 211; Van der Noot, G. W., Reece, H. P., and Skelley, W. C., *J. Animal Sc.*, 1947, **6**, 12; McMillen, W. N., Reineke, E. P., Bratzler, L. J., and Francis, M. J., *ibid.*, 1947, **6**, 305.

16207 P

Observations on Ureteral Peristalsis in Unoperated Dogs.

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Up to the time Trattner[1,2] developed the hydrophorograph for the graphic recording of peristaltic activity in the human ureter, investigations on ureteral peristalsis were carried out on excised ureters, or on exposed ureters *in situ* in anesthetized and operated

1 Trattner, H. R., *J. Urol.*, 1924, **11**, 477.

2 Trattner, H. R., *J. Urol.*, 1932, **28**, 1.

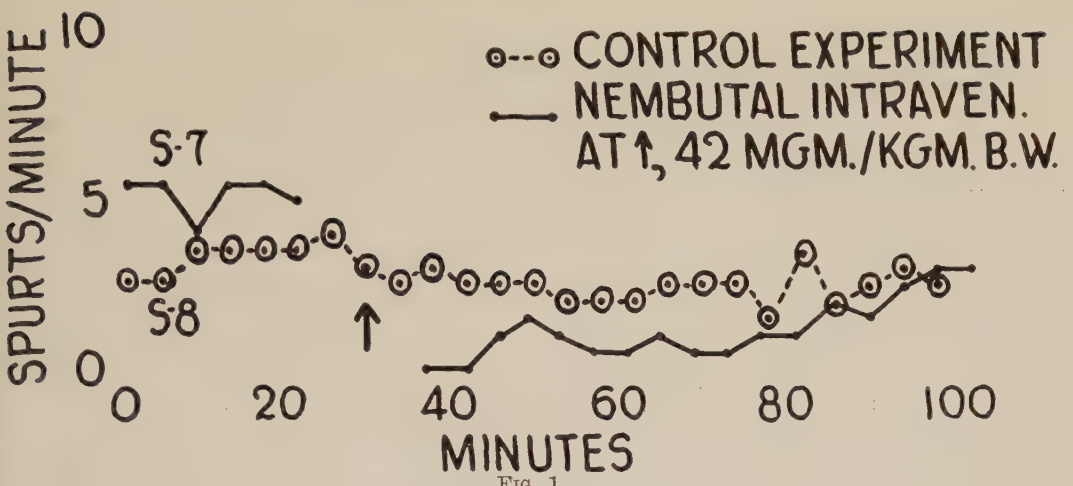

FIG. 1.
Ureteral Peristalsis in Unoperated Dog.

animals. Trattner's method resulted in a more physiologic approach to the study of ureteral peristalsis in that anesthesia and operative manipulation were eliminated. The present work likewise eliminated all operative trauma, and also anesthesia except in those experiments in which the effects of the anesthetic *per se* were being investigated. It goes one step further to realize the ideally physiologic experiment in that no catheter is introduced into the ureter. Although the presence of a catheter in the lower end of the ureter may have very little, if any, effect on the activity of the upper part of the structure, the absence of the catheter is a closer approach to the normal state.

Materials and Procedure. Well-trained female dogs, weighing approximately 11 kilograms, were loosely restrained lying supine on a dog-board. The animals had been without food for 24 hours but had free access to water. The urinary bladder was catheterized with a hard-rubber catheter and was washed free of urine with distilled water. This preliminary catheterization dilated the urethra so as to facilitate the passage of the cystoscope (No. 16 Fr. children's cystoscope). After several washings of the bladder the catheter was removed and the cystoscope introduced into the bladder without difficulty. No local anesthetic was used. One cubic centimeter of a sterile 4% aqueous solution of indigo carmine was injected intramuscularly. Three to fifteen minutes later the ejection of the dye from the ureteral orifice was observed. Although only one ureteral orifice could be watched because of the limited visual field of the cystoscope, in a few experiments the 2 ureteral orifices were so located that the spurting of dye from both could be observed simultaneously. (The curves and the table give data for one ureter in each experiment, however.) The bladder was always distended with about 50 cc of water to facilitate the observations of the spurts of dye.

After the dye entering the bladder became distinctly visible, the number of spurts of dye was counted, usually for a 2-minute period, followed by a 2-minute rest period for the observer. This alternation of counting and rest periods was continued throughout the experiment. If the dog was restless the counts were made for one minute, followed by a 2-minute rest period. The dye was frequently washed from the bladder with distilled water through the cystoscope, the water entering from a reservoir suspended above the animal. Only the observers who began the experiment and who were known by the animals were allowed in the room during the experimental period on the unanesthetized animals so as to minimize emotional disturbance of the animals.

The cystoscopic observation of dye entering the urinary bladder of unanesthetized dogs was reported by Milliken and Karr[3] in their studies

[3] Milliken, L. F., and Karr, W. G., *J. Urol.*, 1925, **13**, 1.

TABLE I.
Effects of Anesthesia on Ureteral Peristalsis.

Experiment No.	Ureteral peristaltic activity—per minute				
	Pre-experimental		Experimental (Intravenous nembutal)		
	Period 1	Period 2	Period 3	Period 4	Period 5
S-7 (42 mg/kg)		5.6 (4.5-6.0)	1.2 (0.5-2.0)	1.1 (1.0-1.5)	2.1 (1.5-3.0)
S-5 (30 ")	4.2 (3.5-6.5)	4.3 (3.5-5.5)	4.7 (3.5-8.0)	4.1 (3.5-4.5)	4.0 (3.5-4.5)
S-4 (29 ")	4.8 (3.0-6.5)	5.0 (3.0-6.0)	2.2 (2.0-2.5)	2.7 (2.5-3.0)	2.9 (2.0-4.0)
S-5 (27 ")		3.8 (3.0-4.5)	2.0 (1.5-2.5)	3.5 (1.5-4.5)	
Controls					
S-8	3.6 (3.0-4.0)	3.7 (3.0-4.5)	2.8 (2.5-3.0)	2.7 (2.0-3.0)	3.2 (2.5-4.0)
S-6	5.7 (4.5-7.0)	4.3 (3.5-5.5)			
S-2	2.7 (2.0-3.0)	3.1 (2.5-4.0)			
S-1	3.1 (2.0-4.0)	2.5 (1.5-3.5)	2.6 (0.5-4.5)		
B-6 (42 mg/kg)		4.6 (4.0-5.0)	5.8 (5.0-8.5)	4.2 (3.5-5.0)	3.9 (2.5-4.5)
B-3 (28 ")		5.7 (5.0-7.0)	4.2 (3.5-5.0)		
B-5 (25 ")	4.7 (4.0-5.5)	7.2 (6.0-9.5)	4.9 (3.5-6.0)	3.9 (3.5-5.0)	
B-4 (25 ")		4.7 (4.5-5.0)	5.3 (3.0-10.5)	4.6 (4.0-6.0)	4.1 (3.0-5.0)
B-2 (25 ")		4.9 (4.5-5.5)	3.5 (2.5-5.0)		
Controls					
B-7	7.4 (5.0-9.0)	8.9 (6.5-10.0)	7.2 (4.5-10.0)	6.2 (5.5-7.0)	
B-1	4.0 (3.0-4.5)	3.4 (2.0-4.5)			
Q-3 (31 mg/kg)		3.9 (2.0-5.0)	4.3 (3.0-10.0)	2.7 (2.0-4.0)	3.8 (3.0-5.0)
Q-4 (29 ")	6.2 (5.5-7.0)	5.8 (5.0-7.0)	4.6 (0.0-6.5)	5.5 (5.0-6.0)	6.0 (5.0-7.0)
Controls					
Q-2	3.2-(3.0-5.0)	3.6 (3.0-5.0)			
Q-1	2.9 (2.5-3.0)	3.0 (3.0-3.0)			

The numbers given in the columns under Period 1, Period 2, Period 3, etc., are the averages usually of 5 or 6 two-minute periods for a 20-minute duration. The figures in parentheses give the range of peristaltic activity for that period. Between Period 2 and Period 3, the nembutal was injected and after a short delay the counts were again begun.

on the influence of renal sympathectomy on kidney functions, but a study of ureteral peristalsis *per se* under various physiologic and experimental conditions was not carried out by these authors.

Results. The responses of the ureters in 2 experiments are plotted in Fig. 1. Experiment S-8 is a control experiment on an unanesthetized dog and shows the relative constancy of ureteral peristalsis over 100-minute period. Experiment S-7 shows the effect of a single rapid intravenous injection of pentobarbital sodium (nembutal). Peristaltic activity was reduced from an average of 5.6 spurts per minute to 1.1, followed by a gradual recovery.

The results of 11 experiments with varying doses of intravenous nembutal and eight control experiments are summarized in Table I. The 19 experiments were performed on 3 dogs.

Conclusions. Observations of ureteral peristalsis can be made on unoperated and unanesthetized dogs and conclusions drawn as to the activity of the ureters. Although the series is small, results suggest that nembutal inhibits ureteral peristaltic activity. The mechanism of this inhibition may be through the influence of the anesthetic directly on the ureter; or the anesthetic may affect the urine production and thus the volume of urine passing down the ureter, since variations in urine volume will affect peristalsis of the ureters.

16208 P

Inhibition of Typhus and Spotted Fever by Intradermal Inoculation of Antiorgan or Certain Normal Sera.*

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The present report includes one phase of the study on the mechanism of action of antiorgan sera (ACS=REIS) on infectious processes,[1] namely: on the course of typhus and spotted fever† in guinea pigs after intradermal inoculation into an area previously infiltrated with the antiserum. This involves primarily the role of the skin as a natural portal of entry and as dermal barrier for these infections.

Methods. A square area (1 cm along each side) on the shaved abdominal skin of the guinea pig is delineated and injected intradermally at 4 corners toward the center with the serum in 1:5 or 1:10 dilutions (0.1 ml at each corner). The center of this area is injected 2 hours later with 0.1 ml of a 10% brain suspension of a typhus or spotted fever guinea pig (Anigstein *et al.*[2]) The antiorgan serum was prepared in rabbits with guinea pig spleen and bone marrow as antigen according to the original technic of Marchuk,[3] and its modifications (Anigstein *et al.*[4]). Each series of test guinea pigs was accompanied by controls injected either with infective brain tissue alone, or in addition, with normal rabbit serum substituted for the immune serum. The potency of the antisera was based on complement fixation titers with homologous antigen (spleen and bone marrow), and by action on the outgrowth of tissue explants (Pomerat[5]). In one series, the globulin fraction of the antiguinea pig serum in dilution 1:10 was used. Absorption tests with boiled sheep cells of both antiorgan and "normal" rabbit sera showing antisheep hemolysins were carried out to study the possible role of the Forssman antibodies. Groups of 6 to 12 afebrile guinea pigs of both sexes (400-500 g body weight) were used in each series of experiments involving various combinations of the test sera. Body temperatures of test guinea pigs were recorded daily over a period of 18 days.

Results. The first series of 6 guinea pigs were treated with antiguinea pig rabbit serum (dilution 1:10) and 2 hours later injected at the site of serum inoculation with the brain suspension of a typhus infected guinea pig. Of these, 4 animals remained afebrile; in one the course of the disease was markedly attenuated, and one showed typical typhus. All controls reacted with typical fever after 6 days incubation. Of 6 test animals in another series, 5 remained afebrile, while only one responded with fever of 2 days duration. Significant results were obtained with antiorgan serum No. 91 (complement fixation titer 1:150; hemolysin titer 1:1000) used in a dilution 1:5. Of 5 guinea pigs, 4 remained afebrile, one developing fever after 11 days. The controls treated with serum (diluted 1:5) from the same rabbit (No. 91) bled before immunization ("normal" serum), reacted with typical typhus.

Occasionally, sera from untreated ("normal") rabbits attenuate the course of the

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† The strain of louse-borne typhus was secured through the courtesy of Dr. John P. Fox, Laboratories of the International Health Division, Rockefeller Foundation, New York. The Rocky Mountain spotted fever strain was kindly supplied by Dr. R. R. Parker, Director, Rocky Mountain Laboratory, U. S. Public Health Service.

1 Anigstein, L., and Pomerat, C. M., *Tex. Rep. Biol. Med.*, 1945, **3**, 545.

2 Anigstein, L., *et al.*, *J. Immunol.*, 1944, **48**, 69.

3 Marchuk, P. D., *Am. Rev. Sov. Med.*, 1943, **1**, 113.

4 Anigstein, L., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 279.

5 Pomerat, C. M., unpublished data.

infection, showing abortive fevers of one to 2 days duration, in contrast with the afebrile course of the animals treated with the antiserum. The serological analysis of these "normal" sera gave evidence of lysins against sheep cells with titers of 1:20 or 1:40, and in some cases as high as 1:200. This has been recorded by other workers and attributed either to constitutional genetic factors (Witebsky and Neter[6]), or to past infections of heterogeneous origin (Hyde[7]). Attempts to investigate the possible role of the hemolysins by their absorption from both normal and immune sera with boiled sheep cells do not suggest correlation of heterophile antibodies with the observed protective action.

Experiments with guinea pig antiserum No. 91 also showed protective qualities when challenged with the highly virulent spotted fever, namely, abortive fever reactions, or complete absence of fever were observed. No greater protection was afforded by the globulin fraction of antiguinea pig serum (Complement fixation titer 1:300; hemolysin titer 1:1250) used in dilution 1:10. Slight elevations of body temperatures not exceeding 40°C for 1-2 days were recorded in 5 guinea pigs of the 10 used in the series. The temperatures of the other 5 animals remained normal over the 18 days observation. On the contrary, the 3 untreated controls developed typical spotted fever. As observed in the typhus series, the course of spotted fever was occasionally attenuated by certain "normal" rabbit sera.

Conclusions. Antiguinea pig rabbit immune sera, when injected intradermally into guinea pigs inhibited the clinical manifestations of typhus and spotted fever after the infective material was inoculated at the site of the serum administration. Highly protective properties against typhus and spotted fever, previously absent in the serum of untreated rabbits, were demonstrated in the antisera of the same animals after their immunization with guinea pig spleen and bone marrow. However, an attenuation of typhus and spotted fevers occurred when serum of certain "normal" rabbits was used. The presence of Forssman antibodies in the rabbit immune sera and of sheep cell lysins in the "normal" sera seems of no significance. Among the factors involved in the observed phenomena, the barrier effect of the skin and its modified permeability are probably decisive. The role of the spreading factor in the invasiveness of rickettsiae is under systematic experimental study.

The authors make no suggestions as to the possibilities of utilizing the methods and results described as a practical application for protection against or treatment of typhus or spotted fever.

[6] Witebsky, E., and Neter, E., *J. Exp. Med.*, 1935, **61**, 489.

[7] Hyde, R. R., *Am. J. Hyg.*, 1928, **8**, 205.

16209

Autographic Localization of Radio-Iodine in Stained Sections of Thyroid Gland by Coating with Photographic Emulsion.*

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Radioactive materials emit radiations which have the same effect as light on a photographic emulsion. This property has been used to locate radio-elements in histological sections of tissues, and especially radio-iodine in the thyroid gland.[1] The radioactive section is usually placed in contact with a photographic film or plate.[2,3,4] The image or "autograph"

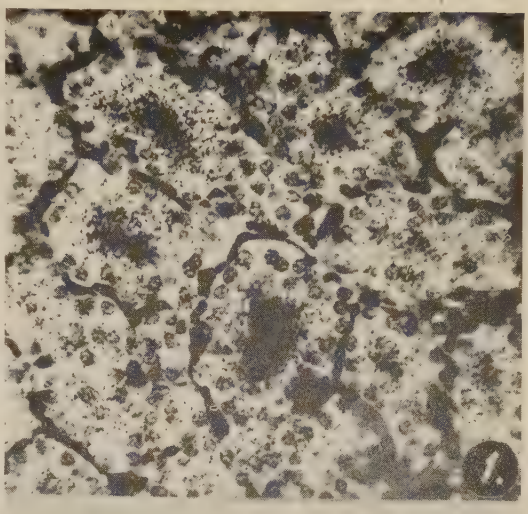
* This work was supported by a grant from the National Research Council of Canada.

[1] Gross, J., and Leblond, C. P., *Canad. Med. Assn. J.*, 1947, **57**, 102.

obtained by this method is rather hazy and cannot be easily compared with the tissue section. These disadvantages are obviated by coating the section with fluid photographic emulsion and staining it after development.[5] A similar modification consists of mounting the sections directly on photographic plates.[6] These technics, however, resulted in erratic staining and some dissolution of silver granules in the staining reagents. The method, was, therefore, modified to permit staining of the section before application of the emulsion. This procedure, described below, results in an autographic image which may be observed under the high powers of the microscope (Fig. 1 and 2).

Technical Details. The thyroids of animals treated with radioactive iodine are fixed in Bouin's fluid or neutral formalin; mercury-containing fluids, such as Susa or Helly, cannot be used. The tissues are embedded in paraffin, sectioned at 3 to 5 micra and stained with either hematoxylin and eosin or Masson's trichrome. The stained sections, dehydrated through graded alcohols, are left for 1 minute in a 1% celloidin solution and dried. In the case of the trichrome stain, however, the slides should be quickly dipped into 1% celloidin for a few seconds, dried and dipped again. All celloidin-coated sections are then dried for at least 6 hours in order to harden the celloidin coating and thus protect the stains from the action of developer and fixer.

Subsequent operations are carried out in the darkroom at 2 to 3 feet from a No. 1 Wratten Safelight. The Kodak medium lantern slide plates used for the preparation of the coating emulsion are sampled for freedom from fog. The plates are soaked in distilled water for 10 minutes at 19° ± 1°C. The softened emulsion is then scraped into a 50 cc beaker, using the edge of another plate as a



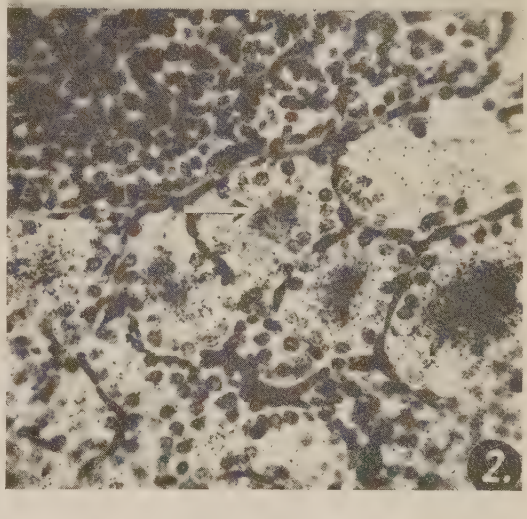


FIG. 1.

Section of thyroid of rat given radioactive iodide. The dark granules of silver, indicative of the presence of radioactivity, are seen in greatest density over the colloid contained in the thyroid follicles. Note that the thyroid epithelium shows a reaction only in the parts of the cells close to the colloid (as a result of the diffusion of the colloid image). The rest of the cell shows only background fog. The black outlines of the follicles represent the deeply staining red blood cells in the vessels. (H. & E., green filter, ×250.)

FIG. 2.

Section of parathyroid (top) and thyroid of rat treated as above. Only a slight background fog is seen in the parathyroid. Thyroid reacts as in Fig. 1. The central arrow indicates a follicle where the colloid may be easily seen through the silver granules. (H. & E., green filter, × 250.)

2 Hamilton, J. G., Soley, M. H., and Eichorn, K. B., *Univ. Calif. Publ. Pharmacol.*, 1940, **1**, 339.

3 Leblond, C. P., *J. Anat.*, 1943, **77**, 149.

4 Leblond, C. P., *Stain Techn.*, 1943, **18**, 159.

5 Belanger, L. F., and Leblond, C. P., *Endocrinology*, 1946, **39**, 8.

6 Evans, T. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 313.

scraper.† One lantern slide plate yields sufficient fluid emulsion to coat 3 or 4 microscope slides. The beaker is then placed in a water bath kept between 38° and 39°C. After 15 minutes in this bath, the emulsion becomes sufficiently liquid for application. A uniform emulsion coating is obtained through the use of a leveling table heated at one end to about 38°C and left at room temperature at the other end. This table was made of a plate glass top, with a heating element under one end, adjusted to give the required temperature. The whole is set on leveling screws and made horizontal with the aid of a spirit level.

The stained slides, after being warmed on the leveling table, are taken in one hand. Four to 8 drops of emulsion are applied with a medicine dropper held in the other hand. The air must be expelled from the dropper before dipping it into the emulsion, in order to prevent the occurrence of bubbles. About 2 drops of emulsion are applied per square inch of slide covered. The drops are spread evenly and quickly with a camel's hair brush. The slide is then rotated from side to side along its long axis to make the emulsion flow gently from edge to edge and thus obtain an even film. This may be seen by the red light reflected from the safelight on the surface of the emulsion.

The slides are returned to the warm side of the leveling table for 30 to 60 seconds and then gently slid to the cool side of the plate. The emulsion gels in about 15 minutes. The slides are then stored at 0° to +2°C in light-tight containers kept dry with P_2O_5. Test slides are developed at various time intervals after coating to determine the proper length of exposure. Development is in Kodak D-72 for 2 minutes at 19° ± 1°C with fixation in acid fixer for 10 minutes. The slides are then washed for 20 minutes, the water being kept below 20°C to prevent buckling and peeling of the emulsion. After passage through alcohol and xylol the sections are mounted in balsam. Five minutes in each bath is necessary for complete dehydration. After placing the coverslips on the sections, these should be left to dry at room temperature. Throughout development and mounting, slides should be kept horizontal.

Discussion. A satisfactory autographic method should permit the visualization of histological details through the silver granules in the gelatin emulsion and thus enable a precise localization of these granules with reference to the stained structures in the section.

This requires the reduction of background fog to a minimum. Normally, unexposed lantern slides will show a certain amount of fog which increases with subsequent manipulations. This increase is avoided by careful control of temperature, minimum safe-light exposure, and storage at 0° to 2°C. In autographs with very little fog, it becomes possible to identify even minute accumulations of silver granules as resulting from the action of the radioactivity. Under these conditions, the length of exposure can be shortened; and the rather small reactions thus obtained make it easier to see the stained structures through the silver granules at the sites of radioactivity.

The photographic reaction due to the β-rays of I^{131} is maximal in the zone located immediately above a site of radioactivity and decreases as the square of the distance from this point. When such a reacting zone is examined through the microscope it appears circular; extending far from the source with prolonged exposure, but being more closely limited to it with controlled exposure. Indeed, with a short exposure, it is possible to limit the image to a zone almost directly above the emittor and thus obtain a nearly cytological localization (Fig. 1 and 2).

Summary. The autographic technic has been modified to permit staining of histological sections before coating with photographic emulsion. With this method, the diffusion of the image is sufficiently decreased to permit examination of the autograph under the high power of the microscope.

† Occasional batches of lantern slides are unserviceable as the emulsion does not soften in water and remains stringy and elastic.

16210

Persistence of the Parabasal Body in a p-Rosaniline Resistant Strain of *Trypanosoma brucei.*

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It has been known for a long time (Werbitzki[1] that certain anti-trypanosomal drugs, *e. g.,* Pyronines, Acridines, Triphenylmethane dyestuffs, elicit a very definite response of the parabasal body of trypanosomes to the effect that this structure can no longer be demonstrated by the usual staining methods such as Wright's, Romanowsky's and Giemsa's stain. It is, moreover, known that trypanosome strains rendered resistant to these drugs may retain the lack of the parabasal body for many years. It was, therefore, surprising to note that in a strain of *T. brucei* which was made resistant to *p*-rosaniline hydrochloride the parabasal body persisted after the parasites had reached maximal resistance to this dyestuff. This rather unique observation seems interesting enough to justify a short description of the preparation and the properties of this drug resistant strain.

The strain of *T. brucei* was kindly given to us by Dr. M. Soule. According to a recent paper by Merchant[2] and a personal communication by Dr. Soule the strain was derived from an original isolation by Bruce (1896) and was kept in guinea pig passages for about 40 years. We became interested in these trypanosomes when it was observed[3] that the organisms possessed a higher sensitivity towards *p*-rosaniline hydrochloride. It is well known that this triphenylmethane dyestuff is of very low activity. Most of the commonly used laboratory strains of *T. equiperdum,*[4] *T. brucei, T. rhodesiense* require a subcutaneous treatment with the maximal tolerated dose (50 mg/kg) or an oral administration of 250 to 500 mg/kg in order to clear the peripheral blood for a period of 3 to 6 days. No permanent cure of the infection can be obtained at least not with a single administration of the dyestuff. An infection of mice with the present *T. brucei* strain responded with permanent disappearance of trypanosomes to a single subcutaneous dose of 50 mg/kg or a single oral dose of 250 mg/kg. Smaller doses down to 10 mg/kg subcutaneously or 100 mg/kg orally cleared the peripheral blood for a 4-6 days period after which relapses occurred. Only doses of 5 mg/kg and 10 mg/kg given by the subcutaneous and oral route respectively failed to show any significant activity.

Drug resistance of this strain towards *p*-rosaniline hydrochloride was obtained by the so-called "short-passage method" described in an earlier paper.[4] Starting with an oral dose of 10 mg/kg the strain became resistant to 500 mg/kg *per os* after 25 to 30 passages. The treatment with this dose of *p*-rosaniline was continued to a total of 47 passages in order to consolidate the fastness. The strain was eventually resistant to 500-1000 mg/kg *per os.* The dose of 1000 mg/kg which is close to the maximal tolerated dose still produced occasionally a more or less marked decrease in the trypanosome content of the peripheral blood, but never cured the animals.

Early in the process of rendering the strain resistant it was noted that the parabasal body persisted in the parasites. Despite the continued administration of *p*-rosaniline and the corresponding increase of resistance towards the dyestuff the parabasal body retained its tinctorial affinity and was easily demonstrated in smears from Giemsa's stain. This is shown in Fig. 1.

This persistence of the parabasal body in *p*-rosaniline-resistant trypanosomes has to our

1 Werbitzki, F. W., *Centralbl. f. Bakteriol.,* 1910 I Orig., 1910, **53**, 303.

2 Merchant, D. J., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 391.

3 Ziering, A., and Buck, M., *E. Ch. Barell,* Jubilee Volume, Basle, 1946, p. 378.

4 Schnitzer, R. J., Lafferty, L. C., and Buck, M., *J. Immunol.,* 1946, **54**, 47.

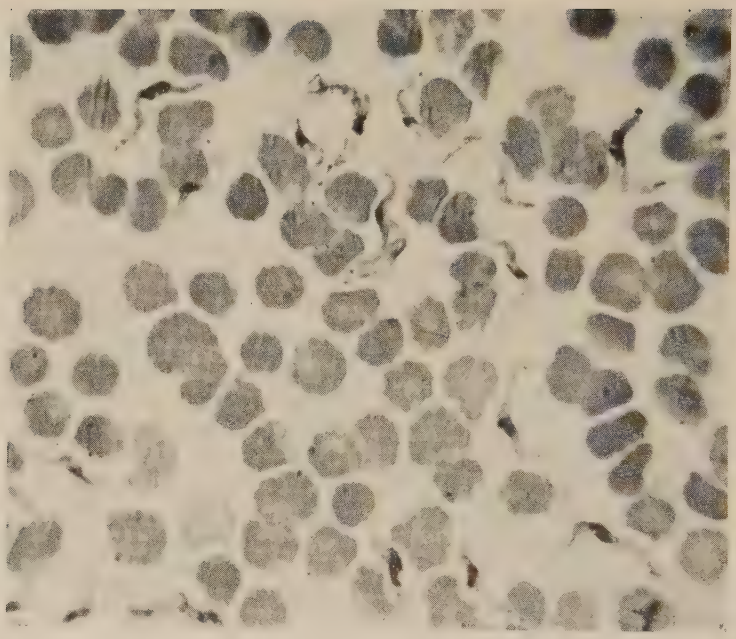

FIG. 1.
Blood of a mouse infected with the *p*-rosaniline resistant strain of *T. brucei*. (47th passage.) Giemsa's stain. Objective apochromate HE 90, compens. eye piece 10 ×. (Photomicrograph by L. Hodax.)

knowledge not been described before. In a number of trypanosome strains rendered resistant to the dyestuff in our laboratory a similar phenomenon was never observed. In particular, of 9 *p*-rosaniline-fast strains of *T. equiperdum* which were prepared in mice and rats and studied during recent years none failed to show the absence of the parabasal body in 80-100% of the individual parasites.

The unusual observation in the resistant strain of *T. brucei* made it desirable to examine the properties of this strain more closely and particularly to investigate its response to different anti-trypanosomal agents.

The results of these specificity tests of the resistant strain are given in Table I.

Mice infected with the parent strain and the *p*-rosaniline-fast trypanosomes were treated with a group of different chemotherapeutic agents of known activity such as 3,3′-4,4′-dihydroxy arseno-benzene-N-methanal sulfoxylate (Neoarsphenamine), 3-amino-4-hydroxyphenyl arsineoxide hydrochloride (Mapharsen), potassium antimonyl tartrate (Emetic tartar), 2,8-diaminoacridinium-10-methochloride (Acriflavin), and a quinoline compound, 6,6′-ureylene bis[4-amino-2-methyl quinoline].

From the data of the minimal active doses, as given in the table, it is evident that the resistance was specific for *p*-rosaniline; the arsenicals, the antimony compound, and the acridine dyestuff showed the same activity on the resistant as on the parent strain. That is characteristic for *p*-rosaniline-fast trypanosome strains.

The observation that the quinoline compound was also active in the infection with the *p*-rosaniline-fast strain was surprising. This compound is a member of a large series of anti-trypanosomal agents[5] and according to previous experience (Schnitzer, unpublished data) proved to be active in experimental infections with normal trypanosomes but without effect on the *p*-rosaniline-resistant modifications of these strains. For instance: Two

[5] Jensch, H., *Angewandte Chemie*, 1937, **50**, 891.

TABLE I.
Sensitivity of the Normal and the *p*-Rosaniline Fast Strain of *T. brucei* Toward Different Anti-Trypanosomal Agents.

Compound	Route	Minimal active dose (mg/kg)	
		Normal strain	Resistant strain
Neo-arsphenamine	subcut.	12.5	12.5
Mapharsen	"	1.0	1.0
Acriflavine	"	5.0	5.0
Emetic tartar	"	5.0	5.0
6,6′-ureylene-bis (4-amino-2-methyl-quinoline)	"	62.5*	62.5*
p-Rosaniline HCl	"	10.0	> 50.0
	per os	100.0	>500-1000.0

* Identical with the curative dose.

p-rosaniline-fast strains of *T. equiperdum** (PR I, PR III) which also lacked the parabasal body no longer responded to subcutaneous treatment with 500 mg of the quinoline derivative per kg, while a tenth of this dose (50 mg/kg) cured the infection with the parent strain.

These observations indicate that an atypical *p*-rosaniline-fast strain of *T. brucei* was obtained. It was characterized by the persistence of the parabasal body and the therapeutic sensitivity to 6,6′-ureylene bis (4-amino-2-methylquinoline). *p*-Rosaniline-resistant modifications of *T. equiperdum* exhibited as a rule the loss of both these properties. The question of the mechanism by which these 2 phenomena, namely, presence or absence of the parabasal body and sensitivity towards 6,6′-ureylene bis [4-amino-2-methyl quinoline] are related or whether they are related at all cannot be decided on the basis of our present experience. We are also not in a position to state definitely that other strains of *T. brucei* might give a similar response to *p*-rosaniline or even that the same strain of *T. brucei* will always react in the same atypical way to attempts of rendering it *p*-rosaniline-fast.

Summary. A *p*-rosaniline-resistant modification of *T. brucei* is described in which—contrary to the experience with other trypanosome strains—the parabasal body persisted. The strain also failed to show the overlapping resistance to 6,6′-ureylene [4-amino-2-methyl-quinoline] which could be demonstrated in *p*-rosaniline-fast strains of *T. equiperdum*.

* The experiments with *T. equiperdum* were carried out by Schnietzer in the Connaught Medical Research Laboratories of the University of Toronto.

16211

Stability of Natural Progesterone.*

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In 1936 approximately one gram of crystalline progesterone was isolated from sows' ovaries for use in the study of some of the biological properties of the hormone.[1] The major portion of this lot was used in the study of various aspects of the hormonal control of

* The authors wish to thank Miss Lila F. Knudsen, of the Food and Drug Administration, Washington, D.C., for her assistance in the statistical analysis of the data.

[1] Allen, W. M., and Goetsch, Carl, *J. Biol. Chem.*, 1936, **116**, 653.

pregnancy in rabbits. During that study no bioassays of the hormone were made to specifically test its stability. The compound was assumed to be stable since the physical characteristics of the crystals did not appear to change with the lapse of time. Small portions of the preparations obtained in 1936, however, were kept for bioassay at a later date to determine their stability. These samples of alpha and beta progesterone have now been assayed and have been found to be as active today, after storage for 10 years, as the first preparations obtained by Wintersteiner and Allen in 1934.[2]

The preparations recently subjected to bioassay were stored during the past decade in small, unsealed, glass-stoppered, vials. The vials were in turn subjected to the temperate environment of a desk drawer without any further attention until the samples were removed for the present experiment.

The melting points of the compound, after 10 years, were essentially the same as originally. The present sample of alpha progesterone was made up of the unused portions of several lots, the original melting points of which ranged between 126° and 129°C. The melting point obtained recently was 123°-124°C. The present sample of beta progesterone melted at 120°-121°C; the original lots from which the present small sample was obtained had melting points ranging from 118°-121°C.

Both the alpha and beta forms were bioassayed according to a slight modification of the original Corner-Allen method.[3,4] Our modified method differed from the original in that ovulation was induced in the rabbits used for the tests by employing commercial chorionic gonadotrophin rather than by mating the animals with the male. Ovulation was obtained by injecting intravenously a freshly prepared aqueous solution of powdered, dry, human chorionic gonadotrophin equivalent to approximately 20 international units. Laparotomy was performed 18 to 24 hours after the administration of the gonadotrophin. At the time of the operation, the ovaries were inspected and if ovulation had occurred, both ovaries were removed and the distal third of one uterine horn resected to serve as a histological control. Beginning on the day of operation and on each day thereafter for a total of 5 days, one-fifth of the total dose of progesterone was administered subcutaneously in 0.2 cc of sesame oil. Twenty-four hours after the last dose, the animals were sacrificed and the uteri removed and fixed in formalin. Transverse sections of the uterine horns were then prepared and stained with hematoxylin and eosin in the usual manner.

The degree of endometrial proliferation was determined by two methods: (a) by comparison with illustrations already published[4] and (b) by measuring the amount of proliferation with a planimeter. The planimetric measurements were made from tracings of the projected image of the section drawn on white paper in the following manner. Three circumscribing lines are drawn in different colors to locate the areas for measurement. Since degree of proliferation is determined as a ratio, the degree of magnification does not need to be kept constant. First, a line (M) is drawn which separates the myometrium from the endometrium. Next, a line is drawn which outlines the outermost aspect of the glands (S). This line divides the endometrium into an outer, unproliferated, non-glandular area and an inner glandular area. This line coincides with the line designated as (M) in some regions of the well proliferated uteri. Finally, a line (L) is drawn separating the lumen from the glands. These lines are separately traced with the planimeter and the circumscribed areas determined. The portion of the endometrium which has become glandular (R) is given by the formula:

$$R\ (\text{response}) = \frac{S - L}{M - L} = \frac{\text{area of glands}}{\text{area of endometrium}}$$

For the purpose of comparison, Table I is compiled from bioassay data obtained in 1934 using the original Corner-Allen method. The sections were re-evaluated without exact

[2] Wintersteiner, O., and Allen, W. M., *J. Biol. Chem.*, 1934, **107**, 321.

[3] Corner, G. W., and Allen, W. M., *Am. J. Physiol.*, 1929, **88**, 326.

[4] Allen, W. M., *Am. J. Physiol.*, 1930, **92**, 174.

TABLE I.
Original Bioassay of Progesterone in 1934 (Corner-Allen Method).
Endometrial Proliferation.

Prep. No.*	Dose, mg	1934	1946†	R	Log dose × 10	θ
			Alpha Progesterone.			
1	1.77‡	4+	4+	.723	1.248	58.3
1	1.33	4+	3+	.816	1.123	64.6
2	1.22	4+	3+	.675	1.086	55.2
3	0.91	3+	3+	.502	.959	45.1
2	0.90	3+	3+	.696	.954	56.6
1	0.88	2+	2+	.544	.944	47.5
2	0.61	1+	1+	.292	.785	32.7
3	0.60	1+	1+	.218	.778	27.9
1	0.44	1+	2+	.313	.643	34.0
			Beta Progesterone.			
2	1.47	4+	4+	.802	1.802	63.6
3	1.28	4+	4+	.784	1.107	62.3
1	1.17	3+	4+	.767	1.068	61.1
2	1.05	2+	2+	.582	1.021	49.7
3	0.94	2+	1+	.402	.973	39.3
1	0.70	2+	3+	.588	.845	50.1
2	0.63	1+	1+	.304	.799	33.4
3	0.63	1+	2+	.357	.799	36.7

* Preparation numbers same as the number from Table I, *J. Biol. Chem.*, 1934, **107**, 321.
† Re-evaluation of the proliferation of the original sections.
‡ Data from animals receiving 1.77 and 1.33 mg of alpha and 1.47 of beta were excluded in the determination of the regression lines.

knowledge of the original interpretations. The estimates of the degree of proliferation of the endometrium were virtually the same as originally.

The bioassay of the samples of alpha and beta progesterone after storage for ten years is recorded in Table II. Several animals were used at each of several levels of dosage, spread over the critical range, to find out how much variation in response is to be expected.

Comparison of the data in Tables I and II shows, first of all, that the quantities necessary to produce full proliferation, and the quantities which produce little or no proliferation, are virtually the same. This indicates beyond doubt that there has been no great change in activity during the interim. Secondly, the variability of response in the critical range of dosage seems to be about the same.

As yet there is no generally accepted method for comparing the activity of compounds having progestational activity with the activity of the international standard of progesterone. Inspection of Tables I and II certainly indicates that the preparations obtained in 1936, and assayed in 1946, have approximately the same activity as the original preparations obtained and assayed in 1934. There is every reason for believing that the results should be similar, since the physical characteristics remained essentially unchanged during the storage period. More detailed comparison of the results of the bioassays, therefore, is justified as it affords in a sense an indication of the reliability of bioassays of identical compounds at widely separated periods of time, even though the results can only be compared with each other and not with concurrent assays of the international standard.

The best approximation of a linear relationship between the dose administered and the response of the endometrium (R—obtained by the planimeter) was secured when the dose was transformed to log dose and the angular transformation† was used on the response. The data for each of the four preparations were subjected to the above transformation and the dosage response curves fitted by the method of least squares. Only tests falling in the critical range (0.4-1.2 mg) were used. The results of this form of analysis of the data are recorded in Table III. The slopes are not very steep, but in all cases they are statistically significant, since in each case the slope

† Fisher, R. A., and Yates, F., *Statistical Tables for Biological, Agricultural, and Medical Research*, Table XII, p. 42, Oliver and Boyd, Edinburgh, 1938.

TABLE II.
Bioassay of Progesterone After Storage for 10 Years (Modified Corner-Allen Method). Endometrial Proliferation.

Dose, mg	Proliferation, Avg	R, Avg	Log dose × 10	θ
		Alpha Progesterone.		
1.6*	4+	.702	1.204	56.9
1.4	4+	.536	1.146	47.1
1.4	4+	.651	1.146	53.8
1.4	4+	.837	1.146	66.2
1.2	3.5+	.741	1.079	59.4
1.2	3.5+	.708	1.079	57.3
1.2	3.5+	.805	1.079	63.8
1.0	2.5+	.650	1.000	53.7
1.0	2.5+	.646	1.000	53.5
1.0	3.5+	.664	1.000	54.5
.8	2.5+	.632	.903	52.6
.8	3.5+	.620	.903	51.9
.8	2+	.581	.903	49.7
.8	2+	.508	.903	45.5
.6	2+	.457	.778	42.5
.6	+	.406	.778	39.6
.6	2+	.461	.778	42.8
.6	+	.373	.778	37.7
.4	0	.182	.602	25.2
.4	+	.450	.602	42.1
.4	+	.476	.602	43.7
.4	+	.433	.602	41.2
.2	0	.143	.301	22.2
.2	0	.193	.301	26.0
.2	0	.158	.301	23.4
		Beta Progesterone.		
1.2	3+	.691	1.079	56.3
1.0	3+	.614	1.000	51.6
1.0	3.5 +	.714	1.000	57.7
.8	1+	.330	.903	35.1
.8	4+	.761	.903	60.8
.8	2+	.541	.903	47.4
.8	2+	.488	.903	44.3
.6	3+	.632	.778	52.6
.6	5+	.273	.778	31.5
.4	+	.438	.602	41.5
.4	0	.026	.602	9.2

* Data from animals receiving 1.6, 1.4, and 0.2 mg were excluded in the determination of the regression lines.

TABLE III.
Analysis of the Dosage Response Curves (log dose — θ response).

Preparation	No. of animals	Intercept of regression line	Standard error of estimate	Slope	Standard error of slope
1934 Alpha	7	50.5	6.02	64.5	15.2
1934 Beta	7	51.6	6.54	73.4	19.1
1946 Alpha	18	54.4	4.69	46.6	6.4
1946 Beta	11	53.8	10.67	66.9	20.7

is more than 3 times its standard error. Likewise the values for the intercept at a dose of 1.0 mg show very little variation. The data indicate, therefore, that the dosage response curves from the data of 1934 do not differ significantly from those obtained from assay in 1946 of preparations that were 10 years old. Likewise there is no significant difference between the curves for alpha and beta progesterone. Using a biological assay procedure with the 1934 data labeled "standard" and the 1946 data labeled "unknown," the potency

thus obtained does not differ significantly from 100%.

Summary. Natural progesterone isolated from pigs' ovaries in 1936 shows no loss of activity after storage for 10 years in glass-stoppered vials at room temperature. Essentially similar methods of bioassay at widely separated intervals of time give dosage response curves that show no statistically significant differences.

16212

Relation of Complement to Blood Coagulation.

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Evidence has been presented both for and against the participation of complement in the coagulation of the blood. Wadsworth, Maltaner and Maltaner[1] favored the affirmative view because many substances were found to have both anticoagulant and anticomplementary actions. Maltaner[2] reported a close correlation between thromboplastic and complement-fixing power of tissue extracts and sera. On the other hand, Wilander[3] and Ecker and Pillemer[4] found great quantitative differences between the relative anticoagulant and anticomplementary properties of a number of substances. Quick[5] showed that complement as a whole is not the same as prothrombin. However, much recent work points to the existence of an additional component of the coagulation system, concerned with the conversion of prothrombin to thrombin. We[6] have already quoted much of this work but should mention in addition the contributions of Ware, Guest, and Seegers[7,8] and the extensive and convincing investigations of Owren.[9] A study of the role that complement plays in the conversion of prothrombin to thrombin appears timely.

Methods. Human plasma was freed of complement activity in 3 ways: by aging and by treatment with zymin and with ammonia. After aging for 2 to 3 months at icebox temperature plasma had no complement activity. Zymin powder was prepared from yeast essentially as described by Ecker, Jones, and Kuehn.[10] Two hundred and fifty milligrams of zymin were mixed with 1 cc of oxalated plasma and 1 cc of imidazole buffer at pH 7.2. Ordinarily serum is incubated with zymin for one hour at 37°C in order to inactivate complement, and smaller quantities than used by us suffice. We found that if the plasma was centrifuged immediately after thorough mixing with zymin, most but not all of the complement activity had disappeared by the time the supernatant was tested, about 20 minutes after the addition of zymin. The remaining complement activity disappeared gradually over a period of one to 2 hours in the absence of zymin. This was the procedure followed in preparing the zymin plasma used in the experiments described subsequently. This

1 Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1937, **33**, 297.

2 Maltaner, Frank, PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 302.

3 Wilander, Olof, *Skandinav. Arch. f. Physiol.*, 1938, **81** (Suppl. 15), 89 pp.

4 Ecker, E. E., and Pillemer, L., *J. Immunol.*, 1941, **40**, 73.

5 Quick, A. J., *J. Immunol.*, 1935, **29**, 87.

6 Mann, F. D., Hurn, Margaret, and Magath, T. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 33.

7 Ware, A. G., Guest, M. M., and Seegers, W. H., *Science*, 1947, **106**, 41.

8 Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

9 Owren, P. A., *The Coagulation of Blood; Investigations on a New Clotting Factor*, Oslo, J. Chr. Gundersen, Boktrykkeri, 1947, 327 pp.

10 Ecker, E. E., Jones, C. B., and Kuehn, A. O., *J. Immunol.*, 1941, **40**, 81.

TABLE I.

One-stage Assays: 0.1 cc of Standard Rabbit Brain Thromboplastin and 0.1 cc of 0.025 M Calcium Chloride Added to Tubes Containing 0.1 cc of Each of the Following Pairs of Components (Total Volume 0.4 cc).

Components present	Complement	Clotting time, sec.
Untreated plasma and saline	Present	19
Zymin plasma and saline	Absent	202
" " " serum	Present	20
NH_3 plasma and saline	Absent	190
" " " serum	Present	22
Zymin plasma and NH_3 plasma	"	32
Aged plasma and saline	Absent	186
" " " serum	Present	21
" " " zymin plasma	"	28
" " " NH_3 plasma	Absent	193

zymin-treated plasma, when mixed with four volumes of fresh oxalated plasma and incubated at 37°C, removed in several hours most of the complement activity of the added plasma. These observations, which suggest that the action of zymin is not one of simple adsorption, are being studied further. Ammonia-treated plasma was prepared by incubating the mixture of 1 cc oxalated plasma, 1 cc imidazole buffer and 0.2 cc of 1% ammonium hydroxide for one hour at 37°C. This mixture had a pH of 8.2 and approximately the minimal concentration of ammonia reported as necessary to inactivate the fourth component of complement in serum.[11] After the treatment with ammonia the plasma was devoid of complement activity. To test for complement 0.2 cc of treated plasma was added to 1 cc of a 1% suspension of sheep cells sensitized with 2 units of amboceptor and the mixture was incubated for one hour at 37°C. Serum was also inactivated by zymin and by ammonia by the same procedures used for plasma. Thrombin-forming activity was tested by a one-stage procedure using a system of 0.4 cc total volume[6] and by a 2-stage procedure,[12] both exactly as previously described except that the plasma was not defibrinated in the 2-stage method.

Results. In all 3 types of complement-inactive plasma, marked loss of thrombin-forming ability was observed for both one-stage and 2-stage methods. The rapid, marked increase in the one-stage prothrombin time on aging is, of course, well known; after prolonged aging, as in these experiments, the yield of thrombin with the 2-stage procedure is also very low. Striking decrease of the one-stage prothrombin time and increase of the 2-stage thrombin yield were obtained with all 3 types of treated plasma by use of 0.1 cc of serum in the 0.4 cc one-stage system and by addition of 0.1 cc of serum to the 2 cc of incubation mixture in the 2-stage procedure. The serum used (4 to 6 hours subsequent to spontaneous coagulation) did not clot fibrinogen and contained no fibrinogen and only very small amounts of prothrombin as shown by the 2-stage assay. Serum inactivated with zymin or ammonia did not restore thrombin-forming activity to similarly inactivated plasma. Mixtures of zymin plasma and aged plasma formed thrombin readily and had appreciable, although not normal, complement activity. Mixtures of aged plasma and ammonia plasma showed no restoration of thrombin formation and little or no complement activity. The addition of serum inactivated by heating at 56°C for 30 minutes partially restored complement activity to ammonia-treated plasma but did not restore thrombin-forming ability. An illustrative protocol (Tables I, II, and III, all data obtained with the same reagents on the same day) shows most of the effects observed. All of the foregoing experiments were easily reproducible.

Comment. The observation that plasma, by 3 different methods, may be almost completely deprived of its ability to form throm-

[11] Pillemer, L., Seifter, J., and Ecker, E. E., *J. Immunol.*, 1941, **40**, 89.

[12] Hurn, Margaret, and Mann, F. D., *Am. J. Clin. Path.* (Tech. Sect.), 1947, **17**, 741.

TABLE II.

Two-Stage Assays: Dilution of Various Plasmas Is That in the 2 cc of Incubation Mixture Containing Calcium and Thromboplastin. Units of Prothrombin Are Calculated in Terms of Original Plasma or Total Plasma When Aged Plasma Is Included. Amount of Serum Is That Added to the 2 cc Incubation Mixture. The Very Low Yields of Thrombin Give Clotting Times Too High for Accurate Determination.

Plasmas and dilutions		Serum, cc	Yield of thrombin, units per cc of plasma
Untreated	1:100	0	490
None		0.1	< 5
Zymin	1:40	0	Slight trace
,,	1:40	0.1	280
NH_3	1:40	0	<20
,,	1:40	0.1	290
Zymin	1:80 + NH_3 1:80	0	320
Aged	1:40	0	<20
,,	1:80	0.1	300
,,	1:80 + Zymin 1:80	0	300
,,	1:80 + NH_3 1:80	0	<20

N.B. Units of prothrombin are determined in terms of yield of units of thrombin.

TABLE III.

Tests for Complement Activity: 1 cc Sensitized Sheep Cells Added to Each of the Following.

Components present	Hemolysis
.1 cc serum + 0.1 cc saline	Complete
.1 ,, untreated plasma + 0.1 cc saline	,,
.2 cc zymin plasma	None
.2 ,, NH_3 ,,	,,
.2 ,, aged ,,	,,
.1 ,, zymin ,, + 0.1 cc NH_3 plasma	Partial
.1 cc zymin plasma + 0.1 cc aged plasma	,,

bin while leaving abundant prothrombin which is highly reactive in the presence of the proper conversion factors, makes it somewhat doubtful whether any method of assay yet devised measures a single substance, prothrombin. The foregoing experiments point to a complex rather than a simple prothrombin conversion factor.

The inactivations of the third and fourth components of complement by zymin and by ammonia respectively are regarded as fairly specific, being in fact the bases for definition of these components. To regard the blood coagulation system and complement as closely related appears to be the point of view most likely to direct efforts toward increasing knowledge of both.

Summary. Inactivation of the complement of plasma by aging or by treatment with zymin or with ammonia blocks conversion of prothrombin to thrombin while leaving ample reactive prothrombin.

16213

Cultivation of Toxoplasma in Embryonated Egg. An Antigen Derived from Chorioallantoic Membrane.

JOEL WARREN AND SUDIE B. RUSS. (Introduced by Joseph E. Smadel.)

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Neutralization[1] and complement-fixation[2] tests have been used for establishing the diagnosis of toxoplasmosis in man; however, neither method is entirely satisfactory. The

1 Sabin, A. B., and Ruchman, I., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 1.

2 Warren, J., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 11.

former requires the use of living parasites and test animals, whereas the rabbit brain antigen, generally used in the latter, gives non-specific reactions under certain conditions. The present report describes the successful cultivation of toxoplasma parasites in embryonated eggs together with a method for preparing complement-fixing antigen from infected chick embryo tissue. Preliminary diagnostic data obtained using this antigen in tests with sera of patients are included.

Materials and Methods. (a) *Strain of Toxoplasma and Its Maintenance in Embryonated Eggs.* The RH strain of toxoplasma of human origin[3] was employed in the present studies. Infected mouse brain material was inoculated into embryonated eggs and the agent subsequently was maintained by serial passage in the avian host. For this purpose the brains of those embryos which appeared moribund 7 to 8 days after inoculation were removed, and a 10% suspension of tissue, partially clarified by light centrifugation, was injected in 0.1 cc amounts into the chorioallantoic sacs of normal embryos. Impression films, prepared from the brain tissue of selected embryos, were stained by Giemsa's method and the presence of toxoplasma was verified before passage was performed.

(b) *Preparation of Complement-fixing Antigen.* Chorioallantoic membranes bearing macroscopic lesions were harvested from 12 or more eggs on the seventh day after inoculation. These were pooled, ground in a mortar with sterile alundum and a 10% suspension was prepared by adding physiological saline solution buffered at pH 7.4. This crude suspension was frozen and thawed 3 times and then clarified by centrifugation at 3,500 r.p.m. for 15 minutes in an angle machine. The supernatant fluid, after addition of sufficient merthiolate to bring the concentration to 1:10,000, constituted the antigen. This was generally stored at –20°C until used. Normal 16-day-old chick embryos were similarly treated to prepare an antigen for use as control material.

(c) *Specific Immune Sera.* Immune sera were obtained from rhesus monkeys and from guinea pigs convalescent from infection with toxoplasma. Monkeys were infected by the subcutaneous inoculation of 0.1 cc of mouse brain suspension containing approximately 10,000 to 100,000 minimal lethal doses of the organism as estimated by intracerebral titration in mice. Guinea pigs were inoculated intraperitoneally with 100 to 1,000 minimal lethal doses of toxoplasma similarly estimated. Both monkeys and surviving guinea pigs were bled for immune serum approximately 30 days after inoculation.

Sera from a number of patients who had been suspected of having a toxoplasma infection were available for study. These sera had been stored at –20°C for from a few days up to 3 years.

(d) *Neutralization Tests.* The presence in serum of neutralizing antibodies against toxoplasma was determined by the method of Sabin and Ruchman.[1] In this procedure mixtures of serum and infectious mouse brain suspension are inoculated intracutaneously into rabbits and the resultant lesions are read on the fourth to seventh day.

(e) *Complement-fixation Tests.* Tests for complement-fixing antibodies were performed in the following manner: Tubes containing 0.25 cc amounts of the appropriate dilutions of the serum to be tested received 0.25 cc of antigen (4 units) and 0.5 cc of fresh diluted guinea pig serum containing 2 units of complement as determined by preliminary titration in the presence of four units of antigen. These were incubated overnight at 5°C. The hemolytic system, consisting of 0.5 cc of an equal mixture of a 3% suspension of washed sheep erythrocytes in saline and diluted amboceptor (2 units), was then added to the tubes. The mixtures were incubated at 37°C for a half hour and then read in the usual manner. In addition to the usual controls, each serum was tested with normal antigen employed at a dilution comparable to that of the toxoplasma antigen.*

Experimental. (a) *Cultivation.* The introduction of toxoplasma into the chorioallantoic, amniotic, or yolk sacs almost invariably re-

[3] Sabin, A. B., *J. A. M. A.*, 1941, **116**, 801.

* All complement-fixation tests reported in this paper were performed by Mr. M. J. Snyder of this department to whom the authors wish to express their gratitude.

TABLE I.
Distribution of Toxoplasma in Embryonic Tissues.

Egg passage No.	Days after infection	Intracerebral infectivity titer of tissue for mice				
		Emb. brain	Emb. torso	Allant. memb.	Allant. fl.	Yolk sac
1	7	$10^{-4.4}$	$10^{-4.5}+$	$10^{-4.5}+$	—	$10^{-4.0}$
2	7	$10^{-2.6}$	$10^{-3.5}$	$10^{-3.3}$	—	$10^{-1.5}$
3	8	$10^{-4.4}$	$10^{-3.5}$	—	$10^{-1.8}$	$10^{-2.5}$
3A	7	—	—	$10^{-5.5}$	—	—
5	8	$10^{-4.6}$	$10^{-3.5}$	$10^{-3.5}$	—	$10^{-2.5}$
11	7	$10^{-4.5}$	$10^{-4.0}$	$10^{-3.8}$	—	$10^{-2.0}$

Embryos were 10 days old at time of inoculation and were incubated at 35°C after infection.

sults in a fatal infection. Growth of the parasite occurs equally well in embryonated eggs which are 6 to 12 days old when inoculated; embryos succumb between the seventh and tenth days after infection with practically all dying on the eighth day. Multiplication of the organism occurs equally well in eggs incubated at 35° or 37.5°C.

Toxoplasma are demonstrable microscopically in all tissues of infected embryos. Tissues and fluids of inoculated eggs have infective titers of $10^{-2.0}$ to $10^{-5.5}$ when tested in mice. However, as indicated in Table I, the highest concentrations of the parasites usually are found in the embryo and allantoic membrane.

The most striking pathological lesions in the infected embryonated eggs are large yellowish-gray nodules, scattered throughout the allantoic membrane (Fig. 1). These may be 2 mm in diameter and are frequently visible through the unbroken shell upon transillumination. The embryos themselves are deeply hemorrhagic and occasionally show nodular lesions in the viscera or the skin. The nodules on microscopic examination consist of a central area of necrosis surrounded by a zone of mononuclear leukocytes and contain many toxoplasma scattered throughout the necrotic and infiltrated portions of the lesion.

(b) *Complement-fixing Antigen.* Antigens prepared from infected chorioallantoic membrane were compared in complement-fixation tests with antigens derived by a similar procedure from infected rabbit and mouse brain tissues.[2] Antigens from all 3 sources had titers of 1/8 to 1/16 when tested with 2 units of antibody. The complement-fixing material in the chick embryo preparation had the characteristics of a specific soluble substance since it was separable from the intact organism; data presented in Table II show that no diminution in activity resulted when chick embryo antigens were passed through a Seitz filter or were centrifuged at 14,000 r.p.m. in an angle machine for one hour. Moreover, the antigens are relatively stable; complement-fixing activity was not reduced by lyophilization nor by storage at –20°C for 3 months.

(c) *Preliminary Evaluation of Complement-fixation for Diagnosis of Human Toxoplasmosis.* A number of stored frozen samples of serum were available which had been submitted from adults and children suspected of having toxoplasmosis, all of which had been previously tested for neutralizing antibody. In addition, control sera obtained from "normal" and from syphilitic adults were examined. The above sera were tested for complement-fixing antibodies employing antigen prepared from chorioallantoic membrane. Four of the 11 "normal" sera and 3 of the 14 sera with positive Wassermann reactions fixed comple-

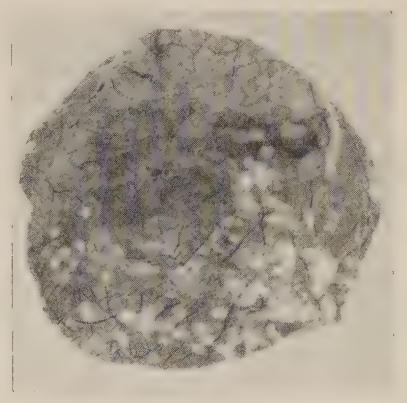

FIG. 1.
Chorioallantoic membrane infected with toxoplasma.

TABLE II.
Complement-Fixation Reactions with Toxoplasma.

Serum used	Serum dilution	Toxoplasma chorioallantoic membrane antigen: Crude suspension 1:2	1:4	1:8	1:16	After desiccation and dehydration 1:2	1:4	1:8	1:16	Seitz filtrate of antigen 1:2	1:4	1:8	1:16	14,000 rpm/60′ 1:2	1:4	1:8	1:16	Normal antigen Crude suspension 1:2
Toxoplasma immune guinea pig	1:25	4+	4+	4+	0	4+	4+	4+	0	4+	4+	+	0	4+	4+	4+	0	0
	1:50	4+	4+	4+	0	4+	4+	3+	0	4+	4+	0	0	4+	4+	3+	0	0
	1:100	4+	4+	+	0	4+	4+	3+	0	4+	4+	0	0	4+	4+	0	0	0
	1:200	4+	3+	0	0	4+	4+	0	0	+	2+	0	0	+	3+	+	0	0
	1:400	0	0	0	0	2+	3+	0	0	0	0	0	0	0	0	0	0	0
Normal guinea pig	1:25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

ment at dilutions of 1/4 to 1/8 with both toxoplasma and normal egg antigens. All of the "normal" sera which reacted in these tests, however, were obtained from individuals immunized with vaccines of egg origin, and fixation of complement by mixtures of normal egg antigen and sera from vaccinated or syphilitic persons is well known.[4,5] By contrast, sera from 47 adults with clinical diagnoses of chorio-retinitis, uveitis, iritis, or visual impairment, all of unknown etiology, were examined. None of these sera had significant amounts of neutralizing or complement-fixing antibody of toxoplasmosis. Tests were also made on sera from 7 children, aged from 6 weeks to 2½ years, on whom a diagnosis of toxoplasmosis was entertained because of 2 or more of the following manifestations: chorio-retinitis, hydrocephalus, cerebral calcification, and mental retardation. Both complement-fixation and neutralization tests were negative with 5 sera and both were positive with the remaining two (infants A and B in Table III). The diagnosis of toxoplasmosis was proved in infant A by recovery of the parasites in inoculated animals and their microscopic demonstration in human autopsy material.

Sera from the healthy mothers of infants A and B both contained neutralizing and complement-fixing antibodies for toxoplasma (Table III). Both types of test were also positive on sera from a third mother who delivered a child with congenital cataracts; serum from the child was not available for study. Negative results were obtained in both types of test with sera from 2 families, mother and child, in which the infant was suspected of having toxoplasmosis on clinical grounds.

The serological results obtained in the case of a 30-year-old male with a peculiar syndrome are worthy of mention. This man (patient F in Table III) had a nodular dermatitis, chorioretinitis, weakness, weight loss, eosinophilia and low grade fever, all of approximately 2 years duration. His serum gave a complement-fixing titer of 1/128 and a strongly positive neutralization test. Repeated attempts to recover toxoplasma from cutaneous lesions,

[4] Wertman, K., *J. Lab. Clin. Med.*, 1945, **30**, 112.

[5] Smadel, J. E., Warren, J., and Snyder, M. J., *J. Bact.*, 1947, **54**, 77.

TABLE III.
Positive Serological Reactions of Patients Having Clinical Evidence of Toxoplasmosis.

Patient	Clinical manifestations	Test	
		Neutralization*	Complement-fixation (Titer)
A	Fatal case of toxoplasmosis in infant	Strongly pos.	1:32
B	Infant with chorioretinitis, cerebral calcification and mental retardation	Positive	1:128
C	Healthy mother of infant A	Strongly pos.	1:128
D	Healthy mother of infant B	Positive	1:128
E	Healthy mother of infant with congenital cataracts	"	1:16
F	Adult with cutaneous nodules, chorio-retinitis and fever	"	1:128

* Neutralization by the test serum of a 1:100 dilution of toxoplasma-infected tissue when the mixture is injected intracutaneously in the rabbit is regarded as a positive reaction; neutralization of a 1:20 dilution as a strongly positive.

blood, and sternal marrow gave negative results. Because of these serological findings, the patient was tested for cutaneous reactivity to toxoplasma material. For this purpose, 1/100 and 1/1000 dilutions of standard complement-fixing antigen and a 1/1000 dilution of normal egg antigen prepared in a similar fashion were injected intracutaneously in 0.1 cc amounts. Although there was no immediate reaction at any of the sites, both of the areas receiving the toxoplasma antigen developed erythema and induration at 24 hours which persisted for several days; the lesion at the site of the 1/100 dilution was 1½ x 2 x 0.1 cm and at the 1/1000 dilution was 1 x 1 x 0.1 cm. No reaction was elicited at the site of the injection of normal egg material.†

Summary. Inoculation of embryonated eggs with toxoplasma resulted in a generalized parasitic disease of this host with a fatal termination. The agent could be maintained by serial passage of embryo brain. Chorioallantoic membranes from infected eggs contained a stable, specific soluble antigen which fixed complement with toxoplasma immune animal serum. Human sera from both proven and suspected cases of toxoplasmosis which were known to contain neutralizing antibody against the parasite also fixed complement with chorioallantoic membrane antigen.

† These studies were performed in collaboration with Drs. A. J. Brennan and T. Brown, Mount Alto Hospital, Washington, D.C., and will be reported in detail elsewhere.

16214

Influence of Oxophenarsine on Hypoglycemic Action of Insulin.

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Noting the results obtained by Barron and Singer[1] in inhibiting the activity of certain enzymes with arsenoxides and their use of this reaction as a test of the essential SH groups of such enzymes, the author thought it of interest to incubate insulin under sterile conditions with the arsenoxide, oxophenarsine (3-amino-4-hydroxyphenyl arsenoxide hydrochloride). While enzyme workers assume that reaction is only with free SH groups, it seemed possible that a part of the arsenoxide might reduce the S-S linkages of insulin to –SH,

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1 Barron, E. S. G., and Singer, T. P., *Science*, 1943, **97**, 356.

being converted to the arsonic acid, and that then another portion might react with insulin –SH to form thioarsenites. It was expected that such a reaction would cause loss of insulin potency. Under the proper conditions rather a prolongation of action but not a loss of potency was observed.

Methods. The insulin used was Lilly Isletin, 20 units/cc. The oxophenarsine hydrochloride was pure 3-amino-4-hydroxyphenyl arsenoxide hydrochloride hemialcoholate† (mol. wt. 254.4), and not the commercial Mapharsen, containing sucrose and sodium bicarbonate buffers.

The insulin bioassay method in preliminary tests was the 5-hour rabbit blood sugar method of Marks.[2] In later tests by the Eli Lilly Co. their standard method of 5-6-hour rabbit blood sugar test was used (tests at 1.5, 3, 5, and 6 hours).

Incubation of insulin and oxophenarsine: Into sterile rubber capped test tubes or bottles under sterile conditions a given volume of the insulin (*e. g.*, 10 cc of 20 units/cc) was delivered and an equal volume of sterile n/320 HCl in 0.85% saline was added. After replacement of the air with nitrogen gas the oxophenarsine salt in proper amount was dropped in, the container sterilely capped, shaken, and incubated for 18 hours at 37°C. The fluid was then used for the dilution (with the same sterile acid saline) to the 1 or ½ unit per 0.5 cc dose injected into the rabbits. Control animals received the same insulin, which in the 1-1 dilution with acid saline was incubated in similar manner without addition of the arsenical (hereinafter called the regular insulin).

Calculation of the molar equivalent of oxophenarsine per molar sulfur content of the insulin: Assuming that 20 units of insulin equalled 2.32 mg of insulin and taking Svedberg's value for the mol. wt. of insulin at 37,000 and the sulfur content at 3.2%, it was calculated that the molecule of insulin contained 37 atoms of sulfur and that a mole of oxophenarsine would be needed for each. (It may be noted that Stern and White's[3] claim of 36 atoms of sulfur, viz., 18 S-S links, would allow for a slight excess of oxophenarsine, while a higher mol. wt. with the same S percentage content would also allow an excess.) By calculation, with an equivalence of one mole of oxophenarsine per 37 atoms of sulfur, 1 mg of insulin would require 0.2534 mg of oxophenarsine salt, and 1 ml of 20 units/ml insulin would require 2.32×0.2534 or 0.5878 mg of the arsenical. Ten ml of 20 units/ml insulin would then need 5.89 mg for one mole, 11.78 mg for 2 moles. To allow a slight excess the above values were rounded to 6.00 mg and 12.00 mg respectively per 10 ml of 20 units/ml insulin.

Results. Preliminary work. An initial test by the Marks method, upon addition of 6.00 mg of oxophenarsine salt per 10 ml of 20 units/ml insulin and incubation, showed an apparent loss of potency from 100% for the regular insulin to 87% for the arsenically treated insulin.

In the expectation of a possible further loss of potency with a doubled quantity of the arsenical, that amount was tried and the incubate tested on 5 rabbits, which, on the first day received the regular insulin (1 unit) and 2 days later received the unknown "treated insulin" (1 unit). This statistically invalid test showed an apparent increase in potency such as might be found in working in the upper flat part of the dose response curve. Hence, another set of 5 rabbits was tested, using ½ unit insulin doses, and again an apparent increase in potency was observed. At this point, however, the 6th hour blood sugars were taken and were found to average 26.1% below the normal at this time.

With the possibility that a delayed reaction insulin had been obtained, arrangements were made with Edward D. Campbell‡ of the Eli Lilly Co. to prepare similar batches of control insulin and oxophenarsin-treated insulin and make a statistically valid test by their standard method.

Results of the Lilly Laboratories test: Based on results obtained from 30 rabbits on

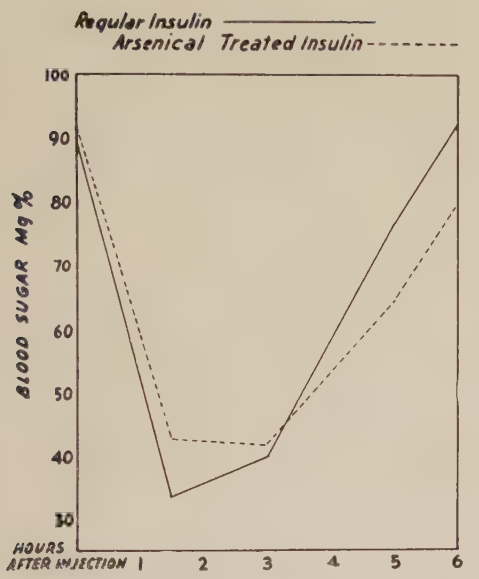
† Kindly supplied by Dr. Oliver Kamm of the Parke Davis Company.

[2] Described in Burn, J. H., *Biological Standardizations, London,* 1938, p. 79ff.

[3] Stern, K. G., and White, A., *J. Biol. Chem.,* 1937, **117**, 95; 1937, **119**, 215.

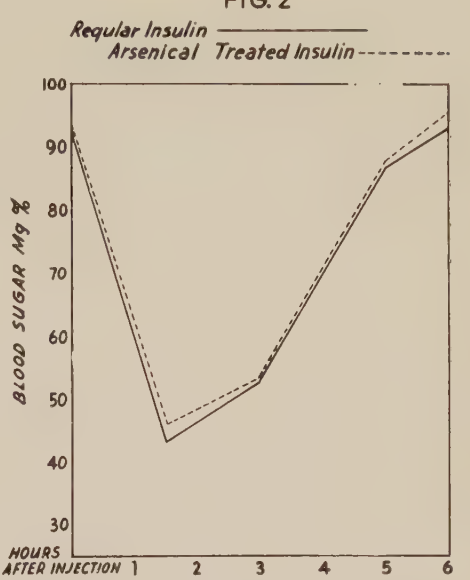
‡ We desire to thank Dr. G. H. A. Clowes and Dr. Campbell for arranging these tests.

FIG. 1



Comparison of the lowering of blood sugar level by oxophenarsine-treated insulin with the lowering induced by regular insulin at the same unit dose. Test conducted immediately after preparation. Avg mg % blood sugar of 30 rabbits for each curve.

FIG. 2



Repetition of the comparison after the arsenical-treated insulin had remained two weeks in the ice-box. Avg mg % blood sugar of 12 rabbits for each curve.

each test (control and unknown) the blood sugar curve shown in Fig. 1 was obtained.

There was no difference in the 2 insulins at the 3rd hour, hence there was no potentiation, but the marked differences at the 5th and 6th hours were typical of an insulin with a delayed reaction.

To test the stability of the As-insulin complex the two specimens were again compared after standing in the icebox for 2 weeks. Two groups of 12 rabbits each were used. The blood sugar curve shown in Fig. 2 was obtained.

The arsenical combination was unstable and at 2 weeks standing in the cold the insulin had regained the hypoglycemic properties of regular insulin.

Discussion. Despite the instability of the oxophenarsine-insulin compound the evidence of interaction may be of interest to those studying the mechanism of action of insulin. It raises the question whether an arsenical interaction with S-S linkages has occurred, such as thioarsenite bridge formation, to delay the normal destruction of insulin by glutathione as postulated by DuVigneaud,[4] or whether interaction might be with free amino groups of the hormone. Hallas-Moeller[5] has reported that reaction of insulin with phenyl-isocyanate produced a delayed reaction insulin claimed to form by a reaction of the isocyanate compound with the histidine and lysine free amino groups. But Stern and White[6] have claimed that acetylation of the free amino groups does not appreciably affect insulin activity, though acetylation of the tyrosine OH groups does. Whatever the mode of reaction may be, the compound formed is easily hydrolysed with regeneration of insulin. A further possibility is a brief, temporary partial inactivation of the insulin. At body temperature this effect may have been decreasing by the third hour, but be sufficient to produce

[4] (a) du Vigneaud, V., Fitch, A., Pekarek, E., and Lockwood, W. W., *J. Biol. Chem.*, 1931, **94**, 233; (b) du Vigneaud, V., *Cold Spring Harbor Symposia Quant. Biol.*, 1938, **6**, 275.

[5] Hallas-Moeller, K., Dissertation, Copenhagen, 1945; *via* author's summary, *Arch. Pharm. og Chemie*, 1945, **52**, 627.

[6] Stern, K. G., and White, A., *J. Biol. Chem.*, 1936, **122**, 371.

a delayed reaction.

Summary. On incubating insulin under sterile conditions in acid saline with 2 moles of oxophenarsine (3-amino-4-hydroxyphenylarsenoxide hydrochloride) for each atom of sulfur contained in the insulin, it was found (by blood sugar determinations) that the effect of the treatment was to prolong the action on the blood sugar beyond that of untreated insulin. However, after 2 weeks standing this delayed action effect had disappeared and the material gave a blood sugar curve identical to that of the control insulin. Possible mechanisms of the interaction of oxophenarsine and insulin are briefly discussed.

16215

Failure to Relate Hyaluronic Acid to Elevated Erythrocyte Sedimentation Rate in Rheumatic Diseases.*

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It has been demonstrated that many asymmetric macromolecular substances are capable of increasing the erythrocyte sedimentation rate (E.S.R.) when added to normal blood. The substances listed by Fahraeus[1] as having such an action were gelatin, agar, gum arabic, gastric mucin, fibrinogen, globulin, and sodium caseinate. In recent years, pneumococcus capsular polysaccharides,[2] desoxyribonucleic acid,[3,4] and hyaluronic acid[4] have been shown to exert a similar effect. Meyer reported[4,5] that a testicular extract containing hyaluronidase, an enzyme complex which depolymerizes hyaluronic acid, when added to the blood of rheumatic fever patients decreased the elevated E.S.R. to normal limits. This report seemed of significance since hyaluronic acid is believed to be a constituent of the amorphous ground substance of the connective tissue, the site primarily involved in rheumatic fever.[6]

The investigation reported here was undertaken to determine whether hyaluronic acid is present in the blood of patients with rheumatic diseases and whether this substance is responsible for the elevated E.S.R. commonly observed.

Materials. The sodium salt of hyaluronic acid was prepared by a modification of the method of Seastone.[7] An aqueous extract of umbilical cord was deproteinized by stirring with a chloroform-amyl alcohol mixture in a Waring blender. The polysaccharide was finally precipitated by the addition of 2 volumes of ethyl alcohol saturated with sodium acetate in the cold. Sodium hyaluronate prepared in this manner produced a clear or opalescent, highly viscous solution at a concentration of 1.0% To avoid discrepancies due to variations in the preparation, one batch was made and used throughout the experiment.

* Supported in part by the Faculty Research Fund of the Horace H. Rackham School of Graduate Studies, University of Michigan.

† Present address: J. S. Youngner, National Institute of Health, National Cancer Institute, Bethesda, Md.; C. H. Altshuler, Department of Pathology, University of Wisconsin, Madison, Wisc.

‡ The authors wish to thank Drs. W. D. Robinson and W. D. Block of the Rackham Arthritis Research Unit for furnishing blood from cases of rheumatoid arthritis and salicylate levels used in this report.

1 Fahraeus, R., *Acta Med. Scandinav.*, 1921, **55**, 1.

2 Nungester, W. J., and Klein, L. F., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 315.

3 Mori, S., *Kekkoku*, 1941, **19**, 50.

4 Meyer, K., Hahnel, E., and Feiner, R. R., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 36.

5 Meyer, K., Chapter 18, *Currents in Biochemical Research*, ed. by Green, D. E., 1946.

6 Klinge, F., *Ergeb. d. allg. Path. u. Path. Anat.*, 1933, **27**, 1.

7 Seastone, C. V., *J. Exp. Med.*, 1939, **70**, 361.

The hyaluronidase§ was obtained from bull testes according to the method of Hahn.[8] The extract was dried while frozen and processed by the Madinaveitia method[9] with ammonium sulfate fractionation. The final enzyme preparation contained 233 turbidity reducing units per milligram.[10]

Methods. Erythrocyte Sedimentation Rate. E.S.R. was measured throughout these experiments by a modified Westergren technic,[11] with no correction for hematocrit.

Estimation of Hyaluronic Acid. Many acid mucopolysaccharides will co-precipitate with protein at an acid pH.[12,13] The resulting turbidity has been used by Kass and Seastone[10] as a quantitative measure of hyaluronic acid; a modification of their procedure was employed. Serial dilutions of sodium hyaluronate were made in normal rabbit plasma, 1.0 ml samples of which were then acidified by adding 9.0 ml of pH 3.1 acetate buffer (0.5 M). Concentration of the sodium hyaluronate in the several samples could be correlated with percent transmission read in a photoelectric nephelometer‖ with a 530 Å filter. In all cases, incubation of the serially diluted sodium hyaluronate with hyaluronidase (1.0 mg in 0.1 ml physiological salt solution) at 37° for one hour prior to acidification completely prevented the turbidity.

An attempt was made to use the carbazole reaction[14] to determine hyaluronic acid colorimetrically. By the direct method of Seibert and Atno,[15] using galactose as the standard, tests on serial dilutions of sodium hyaluronate in normal rabbit plasma showed that the procedure was not sensitive enough to measure accurately the small sodium hyaluronate concentrations needed to increase the E.S.R.

TABLE I.
Effect on Erythrocyte Sedimentation Rate of Adding Sodium Hyaluronate to Oxalated Rabbit Blood and Washed Erythrocyte Suspensions.

Sodium hyaluronate mg/ml	Erythrocyte sedimentation rate	
	Whole blood mm/hr	Washed erythrocytes* mm/hr
0.00	2	1
0.18	6	9
0.37	15	20
0.75	40	50
1.5	80	78
3.0	4†	0†

* Washed 3 times with physiological salt solution, then brought to volume in this diluent.
† Inhibition due to viscosity and clumping effects.

Preliminary Tests. To evaluate the reliability of the methods employed for the determination of hyaluronic acid in blood, experiments were carried out *in vitro* and *in vivo*. The increases obtained in E.S.R. on adding various dilutions of sodium hyaluronate to normal rabbit blood are shown in Table I. The apparent inhibition of the settling velocity in the highest concentration of the polysaccharide (3.0 mg/ml) was due to viscosity and clumping effects, since dilution with whole blood or physiological salt solution (P.S.S.) accelerated the E.S.R. Results similar to those in Table I were obtained with human blood. In every instance, following incubation with hyaluronidase, the settling velocity returned to normal. Hyaluronidase failed to affect the increased E.S.R. produced by gelatin or gum arabic.

In the *in vivo* studies, rabbits weighing 1 to 2 kg were injected intravenously with varying amounts of sodium hyaluronate. Increased E.S.R. and plasma turbidity could be demonstrated for varying periods after injection, as shown in Table II. In every case, the increased E.S.R. and the plasma turbidity were abolished by incubation with 1.0 mg of hyaluronidase at 37° for one hour.

Investigation of Blood from Clinical Cases. Specimens of oxalated blood were obtained

§ Kindly furnished for these experiments by courtesy of the Parke-Davis Company, Detroit, Mich.

8 Hahn, L., *Biochem. Z.*, 1943, **315**, 83.

9 Madinaveitia, J., *Biochem. J.*, 1941, **35**, 447.

10 Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **79**, 319.

11 Westergren, A., *Acta Med. Scandinav.*, 1920, **54**, 247.

12 Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 1936, **114**, 689.

13 Meyer, K., Palmer, J. W., and Smyth, E. M., *J. Biol. Chem.*, 1937, **119**, 501.

‖ Lumetron, Model 400A, Photovolt Corp., New York City.

14 Dische, Z., *Mikrochemie*, 1930, **8**, 4.

15 Seibert, F. B., and Atno, J., *J. Biol. Chem.*, 1946, **163**, 511.

TABLE II.
Intravenous Injection of Sodium Hyaluronate in Rabbits. Inhibition of Increased Erythrocyte Sedimentation Rate and Plasma Turbidity by Hyaluronidase.

Rabbit	Sodium hyaluronate injected, mg/kg	Time after injection	Erythrocyte sedimentation rate after incubation with		Plasma turbidity after incubation with	
			P.S.S.,* mm/hr	Hyaluronidase,* mm/hr	P.S.S. % transmission	Hyaluronidase, % transmission
1	72.2	40 min.	72	1	54	100
		6 hr	15	5	69	100
		96 "	1	—	100	100
2	72.4	3 min	42	1	42	100
		1 hr	53	1	47	100
		6 "	15	1	62	100
3	38.7	30 min	12	1	61	100

* See text.

from 16 cases of rheumatic fever,¶ 16 cases of rheumatoid arthritis, and 10 patients with various other diseases. The initial E.S.R. was first determined. To investigate the effect of hyaluronidase, 1.0 mg in 0.1 ml P.S.S. was added to 1.0 ml of the blood and as a control, 0.1 ml P.S.S. alone was added to an equal volume of blood. These specimens were incubated at 37°C for one hour and the E.S.R. again measured. As indicated in Table III, in most cases where there was a significant alteration of the E.S.R. by hyaluronidase, the P.S.S. alone produced a similar effect.

The turbidimetric method described earlier for estimating hyaluronic acid in plasma was applied to two 1.0 ml samples of plasma from the same blood. To one was added hyaluronidase; to the other P.S.S., as above. After incubation at 37°C for one hour, the plasmas were acidified with the acetate buffer. In no case did the two specimens show a significant difference in the turbidity formed. Rarely was there a measurable difference between the results obtained with the pathologic specimens and those from normal individuals.

It has been reported[16,17] that many blood specimens contain antinvasin, a substance which inhibits hyaluronidase activity. Hyaluronidase inhibition by human or rabbit plasma was not encountered under the experimental conditions in this study.

Many patients involved in this study had been on salicylate therapy. Since this therapeutic agent has been reported to have a hyaluronidase-inhibiting activity,[18,19,20] experiments were carried out to determine whether it had been responsible for the failure of hyaluronidase to alter the E.S.R.

Varying quantities of sodium salicylate (10% in P.S.S.) and sodium hyaluronate were injected intravenously into rabbits, blood was drawn by cardiac puncture at intervals, and salicylate determined by the method of Brodie *et al.*[21] Although salicylate affected the elevated E.S.R. produced by sodium hyaluronate, it did not alter hyaluronidase inhibition of E.S.R. and plasma turbidity (Table IV). This is in agreement with the results of Pike[22] and would seem to rule out salicylate inhibition as a factor in the failure of hyaluronidase to alter significantly the E.S.R. of the clinical blood specimens tested. These findings do not rule out the possibility of interference with the oligosaccharase action of the hyaluronidase

¶ Obtained through the courtesy of Dr. P. V. Wooley of Children's Hospital, Detroit, Mich.

16 Haas, E., *J. Biol. Chem.*, 1946, **163**, 63.

17 Haas, E., *J. Biol. Chem.*, 1946, **163**, 101.

18 Guerra, F., *Science*, 1946, **103**, 686.

19 Guerra, F., *J. Pharm. Exp. Therap.*, 1946, **87**, 143.

20 Dorfman, A., Reimers, E. J., and Ott, M. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 357.

21 Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharm. Exp. Therap.*, 1944, **80**, 114.

22 Pike, R. M., *Science*, 1947, **105**, 391.

TABLE III.
Effect of Hyaluronidase on Elevated Erythrocyte Sedimentation Rate of Blood from Patients with Various Diseases.

Disease condition	Patient	Erythrocyte sedimentation rate		
			After incubation with	
		Initial, mm/hr	Hyaluronidase,* mm/hr	P.S.S.,* mm/hr
Rheumatic fever	1	68	90	83
	2	113	122	115
	3	42	46	37
	4	95	71	90
	5	29	22	14
	6	18	8	7
	7	8	3	2
	8	19	8	8
	9	18	18	30
	10	74	67	55
	11	44	47	—
	12	45	24	16
	13	34	34	25
	14	26	8	10
	15	16	3	3
	16	17	9	5
Rheumatoid arthritis	17	52	34	34
	18	76	79	74
	19	106	122	112
	20	88	62	53
	21	40	40	36
	22	20	12	12
	23	20	15	2
	24	92	86	75
	25	38	42	37
	26	—	35	32
	27	38	33	32
	28	65	52	48
	29	85	80	63
	30	—	72	69
	31	105	107	105
	32	28	29	30
Acute disseminated lupus erythematosus	33	67	55	70
Upper respiratory infection	34	44	41	37
Pulm. tuberculosis	35	30	42	34
	36	62	88	79
Hodgkins disease	37	140	135	127
	38	95	110	95
Lymphosarcoma	39	52	27	27
Aplastic anemia	40	130	140	130
Pernicious anemia	41	41	29	34
	42	59	57	59

* See text.

complex.[23] Salicylates have been reported to decrease the E.S.R. of pathologic blood specimens.[24] The explanation of this phenomenon is obscure and is under further investigation.

[23] Hahn, L., *Arkiv. Kemi. Mineral. Geol.*, 1946, **22**A, No. 1; *Ibid.*, 1946, **22**A, No. 2.

[24] Homburger, F., *Am. J. Med. Sc.*, 1945, **210**, 168.

TABLE IV.
Failure of Sodium Salicylate to Alter Action of Hyaluronidase on Increased Erythrocyte Sedimentation Rate and Plasma Turbidity Following Intravenous Injection of Sodium Hyaluronate.

Rabbit	Sodium hyaluronate, mg/kg	Time after injection	Salicylate level, mg %	Erythrocyte sedimentation rate after incubation with		Plasma turbidity after incubation with	
				P.S.S.,* mm/hr	Hyaluronidase,* mm/hr	P.S.S., % transmission	Hyaluronidase, % transmission
1	91.7	30 min.	37.5	1	1	37	100
		5 hr	16.7	2	2	90	100
2	66.6	1 "	5.6	38	1	48	100
		2 "	4.6	10	1	66	100
3	62.5	1 "	52.8	15	2	52	100
		5 "	33.4	12	2	66	100
4	82.3	1 "	73.8	1	1	53	100
		5 "	43.0	12	1	63	100

* See text.

Discussion. The data presented in this report fail to substantiate the possibility that hyaluronic acid, at least in a polymerized form, is present in the blood of patients with rheumatic fever, rheumatoid arthritis, and several other diseases (Table III) as has been suggested by Meyer.[4,5] The polysaccharide could not be demonstrated in the blood of normal individuals.[25] Depolymerized hyaluronic acid would not be demonstrated by the methods employed here, but previous work[1] and our own indicate that the depolymerized acid will not increase E.S.R. to a marked degree. In a personal communication, Dr. Karl Meyer has stated that in studies with his preparations of hyaluronidase, spreading activity and E.S.R. inhibition did not run parallel. He was able to separate the factor that decreased E.S.R. from hyaluronidase activity by adsorption on lead sulfide.

Summary. 1. Hyaluronic acid is apparently not responsible for the increased erythrocyte sedimentation rate of blood from patients with rheumatic fever and rheumatoid arthritis. 2. Sodium salicylate failed to inhibit the effect of hyaluronidase on elevated E.S.R. produced in rabbits by the intravenous injection of sodium hyaluronate.

25 Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

16216

Immunologic Reactions Following the Intradermal Inoculation of Influenza A and B Vaccine.*

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It has been shown[1] that the intradermal injection of heat-inactivated mumps virus into human beings may exert a pronounced antigenic effect. Data are[2] available which sug-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc. The technical assistance of Sam Lavin and Alice H. Northrop is gratefully acknowledged.

1 Enders, J. F., Kane, L. W., Maris, E. P., and Stokes, J., *J. Exp. Med.*, 1946, **84**, 341.

2 Stokes, J., and Enders, J. F., 1947, unpublished data.

gest that this procedure may also induce active immunity. More recently, Van Gelder and associates[3] compared the rise in antihemagglutinins following intradermal and subcutaneous inoculation of concentrated vaccine prepared from strains of A and B influenza viruses. Although the intradermal dose was only one-tenth as large as the subcutaneous dose, the smaller dose induced a greater mean antibody response. The incidence of generalized reactions was lower in the group which received intradermal inoculations. Localized reactions characterized by redness and swelling were observed in 90% of the subjects on the day following the intradermal injection. Previously, Beveridge and Burnet[4] had noted immediate (10-20 minutes) and delayed (24 hours) dermal responses in certain individuals to intracutaneous injection of inactivated influenza virus. Although in adults they found no correlation between the antibody titer and the delayed reaction, in children the occurrence of the latter appeared to be associated with the presence of antibody.

In the early spring of 1947, an epidemic of influenza appeared imminent in Boston. Intradermal vaccination was then carried out in a group of adult hospital personnel. Information was obtained in respect to the antibody response following vaccination. The occurrence of local and generalized reactions was recorded as well as the incidence of upper respiratory infection following vaccination. These data are presented below.

Materials and Methods. The group consisted of persons working in the Children's Hospital, Boston. On March 19 and 20, 1947, 495 adults were interviewed and a history taken regarding allergic manifestations and previous influenza vaccinations; 24 individuals were eliminated because of a history of severe allergy and 4 because of a history of reaction to previous influenza vaccination. The remainder were given intracutaneously 0.1 cc of a 1:5 dilution in 0.85% salt solution of mixed A and B influenza vaccine (prepared of Lederle Laboratories, Lot No. 2032-34A). Twenty minutes after vaccination, each person was examined and the extent of any erythema and induration at the site of inoculation recorded. Between 44 and 52 hours after vaccination, 449 individuals were re-interviewed, a history obtained of any systemic reaction, and the local reaction was measured. Before vaccination venous blood specimens were obtained from 110 individuals selected at random; 3 weeks after vaccination 88 of these persons were re-bled and questioned as to the occurrence in the interim of attacks of respiratory disease.

Between April 25 and May 5, 1947, questionnaires were filled out by 375 persons concerning the nature of any respiratory disease which occurred subsequent to vaccination. Data were obtained regarding the date of onset, duration, severity, and the presence or absence of the following symptoms: headache, fever, chills, sweating, cough, running nose, pain in eyes, muscle aches, sore throat, and general weakness. During the same period similar questionnaires were filled out by another group of 405 hospital workers who had not received influenza vaccine in March, 1947.

The paired specimens of serum were examined by a modification, developed at the Army Medical School,[5] of the Hirst agglutination-inhibition test using the PR8 strain of influenza A virus and the Lee strain of influenza B virus. Instead of human type "O" cells 0.5% chicken cells were employed.

(1) *Local and Systemic Reactions.* The immediate and delayed cutaneous responses were similar to those described by Beveridge and Burnet.[4] Twenty minutes after inoculation, in those who reacted, a moderate erythema of varying extent appeared around the site with occasionally a small central papule or wheal. The majority also showed after 48 hours a delayed reaction which consisted of a diffusely erythematous and slightly indurated area with a fairly well defined margin; at this time, however, in many persons the reaction had started to subside. The maximum diameters of the local reaction are classified in Table I.

Seventy-five or 16.7% of 449 individuals

[3] Van Gelder, D. W., Greenspan, F. S., and Dufresne, N. E., *U. S. Naval Med. Bull.*, 1947, **47**, 197.

[4] Beveridge, W. I. B., and Burnet, F. M., *Med. J. Australia*, 1944, **1**, 85.

[5] *Bull. U. S. Army Med. Dept.*, 1946, **6**, 777.

TABLE I.
Maximum Diameter of the Immediate and Delayed Cutaneous Responses in 449 Adults Following Intracutaneous Vaccination.

Diameter of erythema, mm	Reaction after 20 min		Reaction after approx. 48 hr	
	No.	%	No.	%
0-4	33	7.3	26	5.8
5-9	43	9.6	40	8.9
10-14	68	15.2	30	6.7
15-19	58	12.9	31	6.9
20-24	64	14.2	35	7.8
25-29	79	17.6	51	11.4
30-34	63	14.1	76	16.9
35-39	22	4.9	43	9.6
40-44	11	2.5	60	13.2
45-49	4	0.9	18	4.0
50-54	2	0.4	22	4.9
55-59	0	0	6	1.3
60-64	1	0.2	3	0.7
65-69	0	0	3	0.7
70 or over	1	0.2	5	1.1
Total reactions > 10 mm	373	83.1	383	85.3

TABLE II.
Frequency of Symptoms Reported by 75 Individuals Who Complained of a Systemic Reaction Within 48 Hours After Intracutaneous Vaccination.

Symptoms	No. of times
Malaise	31
Headache	28
Fatigue	24
Nausea	14
Chills	9
Fever	6
Muscle pain	4
Sweating	1
Urticaria	1

reported that they had experienced a systemic reaction within 48 hours after vaccination. With one exception, the symptoms which are summarized in Table II were very mild. One person requested medical attention because of chills, malaise, and a temperature of 100.3°F.

(2) *Antibody Response Following Intracutaneous Vaccination.* Specimens of serum were obtained immediately before intracutaneous vaccination and again 3 weeks thereafter from 39 individuals who had not been previously vaccinated against influenza and who were free from respiratory disease during this 3-week period. The differences between the pre- and post-vaccination titers are recorded in Table III. From these data it is apparent that the geometric mean of the antihemagglutinin titer rose 3.6 × for Influenza A and 2.9 × for Influenza B following vaccination. If those individuals whose initial titers were over 1:64 are excluded, it has been calculated that the geometric mean titer of 27 persons rose 5 × for Influenza A (1:38 to 1:193) and that of 29 persons 3.2 × for Influenza B (1:28 to 1:89).

Sixteen additional individuals who had reported having a respiratory infection during the period between bleedings showed a rise of the geometric mean titer against Influenza B from 1:38 to 1:134. This group is considered separately because of the presence of respiratory infection in the community, as noted below. However, since there was no evidence of infection with type B virus, there can be little doubt that the rise in titer occurred as a result of vaccination.

(3) *Incidence of Respiratory Disease in the Vaccinated and Unvaccinated Groups.* From the second to the fifth weeks following vaccination there was an increased incidence of respiratory disease in the community. The increase, in part at least, could be attributed to a virus resembling Influenza A as indicated by a significant rise in agglutinin-inhibition titer against the PR-8 strain in the sera of a few unvaccinated patients that were studied and by the isolation of atypical strains of Influenza A virus by other workers in Boston.[6] But analysis of the questionnaires revealed very few cases in which a diagnosis of influenza could be made on the basis of the symptoms reported. Therefore the incidence of all acute upper respiratory infection was determined in the vaccinated and in the control groups. Of 316 individuals who received intracutaneous vaccine and who had not been previously vaccinated against influenza, 109 or 34% reported symptoms of an acute upper respiratory disease; in a comparable unvaccinated control group, 85 or 28% of 329 individuals had an acute respiratory disease.

(4) *Antibody Titer and Local and Systemic Responses to Intracutaneous Vaccination.* No correlation could be discerned between the antibody titer at the time of vaccination

[6] Finland, M., and Morgan, H. R., 1947, personal communication.

TABLE III.

Changes in the Titer of Antihemagglutinins Against Influenza A and B Virus During a 3-Week Period Following Intracutaneous Vaccination.*

Antigen	Reciprocal of agglutinin-inhibition titer	No. of individuals before vaccination	No. of individuals after vaccination	No. showing change in titer						
				Fall	No change	Rise in titer				
						2x	4x	8x	16x	32x
PR-8	<16	0	0	0	0	0	0	0	0	0
	16	7	0	0	0	0	0	3	3	1
	32	6	0	0	0	2	2	1	1	0
	64	14	2	0	0	6	6	2	0	0
	128	8	13	0	2	5	1	0	0	0
	256	2	15	0	0	2	0	0	0	0
	512	2	8	0	1	1	0	0	0	0
	1024	0	1	0	0	0	0	0	0	0
		Total 39								
		Geom. mean titer *1:62*	Geom. mean titer *1:226*							
Lee	<16	6	0	0	0	3	0	2	1	0
	16	8	3	0	0	0	5	3	0	0
	32	7	3	0	2	2	2	1	0	0
	64	8	8	0	2	4	1	1	0	0
	128	8	16	0	4	4	0	0	0	0
	256	2	7	1	0	1	0	0	0	0
	512	0	2	0	0	0	0	0	0	0
		Total 39								
		Geom. mean titer *1:35*	Geom.mean titer *1:104*							

* Previously vaccinated individuals and those with respiratory infection during the serological period were excluded.

and the size of either the immediate or delayed skin reaction. Thus, in 15 previously unvaccinated subjects in whom the agglutinin-inhibition titer against both PR-8 and Lee antigens was 1:32 or less, the average maximum diameter of the delayed skin reaction was 34 mm with extremes of 8 and 50 mm. In 16 similar persons with titers against both PR-8 and Lee of 1:64 or higher, the average maximum diameter was 36 mm with extremes of 10 mm and 60 mm.

Fifty-four of the 449 persons who were inoculated intradermally had previously received influenza vaccine; 39, or 72%, exhibited immediate skin reactions of 20 mm or greater. In contrast, comparable immediate reactions were observed in 205, or 52%, of those vaccinated for the first time. No relationship was noted between the size of the delayed skin reaction and previous vaccination.

No correlation was found between the extent of the delayed skin reaction and the antibody response following vaccination. The average size of the immediate and of the delayed skin reactions did not differ significantly in the group that reported a systemic reaction and in the group that had no systemic response.

Discussion. The results of the tests for changes in antihemagglutinin against Influenza B following intradermal vaccination with inactivated virus showed that when the initial titer of the antibody was low (*i.e.* 1:64 or less), the antigenic effect was appreciable. When the initial titer was moderate or high the response was slight or absent. Inactivated Influenza A vaccine introduced intracutaneously also appeared to exert a comparable, if not a greater, antigenic effect in persons with initial low titers. The fact, however, that an epidemic caused by an atypical Influenza A virus was current at the time this study was carried out prevents a categorical statement in respect to the effect of this antigen since the possible occurrence of inapparent infection cannot be eliminated. Failure of influenza vaccine to stimulate large increases in the antihemagglutinin titer when the initial titer is high has been noted by others after subcutaneous inoculation.[7]

The serologic data indicate that one-fiftieth of the dose of vaccine usually administered by the subcutaneous route when injected into the skin may give rise to antibody levels which are only slightly lower than those reported for the standard subcutaneous dose.[8,9,10,11] In respect to the effectiveness of this route of inoculation our findings are in general in agreement with the observations made by Van Gelder and his associates[3] on influenza vaccine and with those of Enders and his co-workers[1] on inactivated mumps virus. Quantitatively, though, Van Gelder and his associates obtained larger mean increases in titer for both A and B antigens. This difference may possibly be attributed to their employment of undiluted vaccine whereas in our experiments the vaccine was diluted 1:5. Furthermore, they noted that the antibody concentration continued to increase during the period from 2 to 4 weeks after vaccination. Our final determinations were made 3 weeks after inoculation.

In view of the small quantity of virus inoculated the high incidence of systemic reactions which were reported was unexpected. With one exception, however, these reactions caused no loss of working time. It is possible that a large number of very mild reactions were revealed by the method of personal interview which was employed.

Immediate and delayed local erythematous reactions of 10 mm or greater in diameter were encountered in over 80% of the subjects. The immunologic mechanisms underlying these reactions remain obscure. They may be either manifestations of hypersensitivity established by previous experience with the viruses or the effect of a specific toxic action of the vaccines. An unknown proportion of the immediate type might be occasioned by a

[7] Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., *J. Exp. Med.*, 1942, **75**, 495.

[8] Salk, J. E., Menke, W. J., and Francis, T., Jr., *Am. J. Hygiene*, 1945, **42**, 57.

[9] Rickard, E. R., Thigpen, M., and Crowley, J. H., *Am. J. Hygiene*, 1945, **42**, 12.

[10] Eaton, M. D., and Meiklejohn, G., *Am. J. Hygiene*, 1945, **42**, 28.

[11] Hirst, G. K., Plummer, N., and Friedewald, W. F., *Am. J. Hygiene*, 1945, **42**, 45.

non-specific irritative effect of the inoculum.

No evidence for an increased resistance attributable to the vaccination was obtained. The incidence of all types of upper respiratory disease during the 2-month period succeeding vaccination in both inoculated and uninoculated groups did not differ significantly. Several explanations may be offered for the ineffectiveness of the vaccine. The strains of Influenza A may have differed in antigenic composition from the prevailing epidemic strain. This possibility has been invoked to explain the relative ineffectiveness of vaccination in other parts of the country during the epidemic of last spring.[12,13,14] The quantity of vaccine administered may have been too small to establish resistance. Vaccination may have been undertaken too late, since serologic evidence was obtained that Influenza A was present among the hospital personnel within ten days following the administration of the vaccine.

Although the prophylactic value of intradermal vaccination has not been demonstrated, additional experiments would seem desirable in view of the results of Van Gelder and his co-workers and those reported in this communication.

12 Francis, T., Jr., Salk, J. E., and Quilligan, J. J., *Am. J. Public Health*, 1947, **37**, 1013.

13 Sigel, M. M., Shaffer, F. W., and Henle, W., *J. Bact.*, 1947, **54**, 277.

14 Smadel, J. E., *Bull. U. S. Army Med. Dept.*, 1947, **7**, 795.

Summary. 1. Intradermal inoculation into 39 adults of 0.02 ml of concentrated Influenza A and B vaccine gave rise to a mean antibody response (antihemagglutinin) against Influenza B virus which approached that obtained by others following the subcutaneous injection of 1.0 ml of undiluted vaccine. A greater mean increase in antibody against Influenza A was observed in the same group, although this increase could not be attributed conclusively to the effect of the vaccine.

2. The intradermal injection of 0.02 ml of concentrated vaccine into 449 adults was followed within 20 minutes by a local erythematous reaction exceeding 10 mm in diameter in 373 or 83.1%. In 383 or 85.3% of the same group delayed local erythematous reactions exceeding 10 mm in diameter were present after approximately 48 hours accompanied in many cases by slight induration.

3. No correlation could be established between the intensity of the immediate or delayed dermal reaction and the level of antihemagglutinin in the blood.

4. Mild systemic reactions were reported by 75 (16.7%) of 449 vaccinated persons.

5. Although cases of Influenza A infection were demonstrated in the community shortly after vaccination, the rate of all upper respiratory disease during the succeeding 2-month period in a group of 316 vaccinated persons was not significantly different from the rate among 329 unvaccinated persons working in the same institution.

16217

Use of the Spectrophotometer for Measuring Melanin Dispersion in the Frog.

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It is well established that melanophore changes in the frog are related to environmental conditions as well as endocrine factors within the animal. However, there apparently is no satisfactory data on how completely a dispersion or concentration of melanin in the skin will interfere with the reflectance of light by the other pigments present.

A review of the literature regarding methods for measuring melanin dispersion or concentration in the skin indicates that most of the observations made were patterned after

the general descriptive technic reviewed by Parker[1] in which the individual melanophores are given a numerical value corresponding to the various stages of expansion they may happen to be in at the time of examination, and which neglects to consider how effectively the contracted or expanded state of these melanophores will change the appearance of the frog or rest of the pigment normally present in the skin. It should also be pointed out that, even though the results obtained by this method for recording melanin dispersion or concentration in individual melanophores may be accurate and consistent, there always remains an unavoidable subjective factor when one attempts to catalogue melanophores in different stages of expansion in a microscopic field. It would therefore be highly desirable to have an objective method which would give quantitative information concerning the actual amount of masking the dilated melanophores will have over the rest of the skin pigments in the intact animal.

Although Hill and Solandt[2] and Smith,[3] by attaching a photo-electric cell to a microscope, have recorded the variation in transmitted light through the skin and scales of fish under dark and light adapted conditions, they made no attempt to study the effects of melanin dispersion in the intact skin of the frog on the light reflected at each wave length of the visible spectrum. In a search for a method that would answer the requirement above mentioned, it was found that the Hardy[4] photo-electric recording spectrophotometer would successfully give information on the kinds of pigments giving rise to the color of the skin as well as give an objective measurement of the amount of reflectance each pigment may give at its respective wave length when the melanophores are in an expanded or contracted state. The results obtained from experimentation with this technic showed significant differences in skin reflectance in frogs that had previously been either light or dark adapted, and we herewith present the experimental technic used and the results obtained.

In preparing frogs for this type of recording 2 different sets of experimental conditions were used: one group of 8 frogs was exposed to light, and an equal number to dark for the same period of time. To accomplish light adaptation, the frogs were kept for a minimum of 72 hours in a white container which was illuminated at all times by two 40-watt bulbs; for dark adaptation the frogs were kept in a black container for 72 hours, also illuminated by two 40-watt bulbs. To record the skin reflectance the frogs, unanesthetized, were made immobile by fastening them to a frog board. They were then placed so that the back of the frog covered the round aperture (one inch in diameter) of the recording spectrophotometer. The light rays of various wave lengths of the visible spectrum were individually focused on the skin of the frog, and the amount of light reflected in each of the wave lengths was measured and recorded on ruled paper on a percentage reflectance basis. The spectrophotometer was moved by hand in preference to having the complete rotation of the drum made by the motor. The total time necessary for making a complete record was one minute, whereas, with the motor, a total time of 3 minutes was necessary. To insure accuracy the frogs were always placed in the same position before the light aperture of the instrument and the time of exposure was kept constant to avoid any inconsistencies so far as light from the spectrophotometer and melanophore responses were concerned.

The reflectance from the skin surface of the light adapted frogs is definitely greater in all wave lengths measured than the reflectance from the skin of the dark adapted frogs. This can be seen in the graph, which shows the average % reflectance at various wave lengths for light (upper curve) and dark (lower curve) adapted frogs. The results also indicate that melanophore expansion is apparently able to mask the reflecting power of the other pigments in the frog skin. It is

1 Parker, G. H., *Biol. Bull.*, 1943, **84**, 273.

2 Hill, A. V., and Solandt, D. Y., *J. Physiol.*, 1934, **83**, 13.

3 Smith, D. C., *J. Cell. and Comp. Physiol.*, 1936, **8**, 83.

4 Hardy, A. C., *J. Optic. Soc. America*, 1935, **25**, 305.

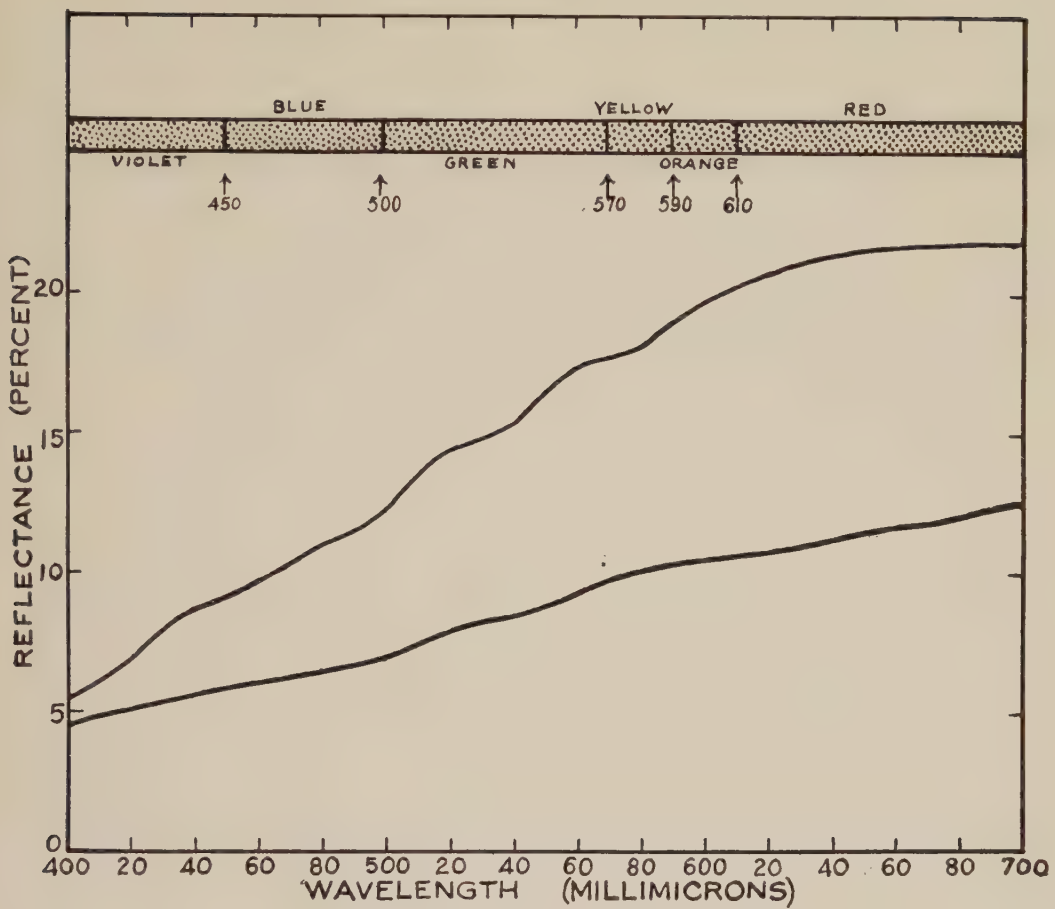

FIG. 1.

interesting to note that the general shape of the curve and degree of reflectance from the skin of the frog under these conditions compare quite closely with the degree of melanin distribution and concentration obtained by Edwards and Duntley[5] from the dorsal surface of the hand of a Negro when examined with the same technic as used in these experiments. Furthermore, the reflectance from the skin surface of the frog is greater for wave lengths at the red end of the spectrum than at the violet end. Statistical analysis of the data justifies these conclusions. Although it is obvious from the data that there is a definite difference in reflectance between light and dark adapted frogs, it is rather surprising to find that the light adapted frogs reflect an average of only 21.8% in the red end of the spectrum where the actual reflectance is at its maximum, while the dark adapted frogs, black as they appear to the eye in comparison, reflect an average of 12.3% at the same wave length. Surely these quantitative measurements show the inadequacy of our subjective observations.

In a few additional experiments, it was interesting to note that frogs made permanently pale as a result of complete hypophysectomy[6] showed melanophores that did not constrict so much or allow for so great a reflectance as those in frogs that were light adapted. If the frogs were made permanently dark because of melanophore expansion following injury of the pars tuberalis region of

[5] Edwards, E. A., and Duntley, S. Q., *Am. J. Anat.*, 1939, **65**, 1.

[6] Hogben, L. T., *Quart. J. Exp. Physiol.*, 1923, **13**, 177.

the pituitary gland,[7] the dilated state of the melanophores was greater and showed definitely less reflectance than frogs that were dark adapted by environmental stimulation only. Further studies of the same kind in which various dosages of related hormones are given may lead to helpful information concerning the mechanism of chromatophore action in animals.

Summary. (1) The spectrophotometer has been used as an instrument for recording the effects of melanophore expansion on the reflecting power of the skin pigments of the frog. (2) The reflectance in the light adapted frogs may be as much as 10% above that of the dark adapted frog at the same wave length. (3) Melanophore expansion in dark adapted frogs masks the reflecting power of the skin pigment at the red end of the spectrum more than at the violet. (4) The average skin reflectance in the light adapted frogs is not more than 21.8%. (5) A difference between the response of melanophores to environmental conditions and operations of the hypophysis is suggested.

[7] Steggerda, F. R., and Soderwall, A. L., *J. Cell. and Comp. Physiol.*, 1939, **13**, 31.

16218

Interrelationship of Vitamin D and the Sex Hormones in Calcium and Phosphorus Metabolism of Rats.

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Introduction. The present therapeutic trend for treating chronic arthritis in humans involves the use of high dosages of vitamin D, and has many successes to its credit.[1,2] The favorable treatment of Raynaud's disease in the same manner, in conjunction with sex hormones, has been reported.[3] The high incidence of arthritis in women at a time near to or following the menopause, and the more gradual appearance of this disease in men, is well known. This leads to the suggestion that the role of the sex hormones in these relationships involving the therapeutic use of vitamin D should be more fully understood.

Hypertrophy of the parathyroids has been described in a number of suggestively analogous conditions, viz.: rickets,[4] scleroderma,[5,6,7] Raynaud's disease,[8,9] and chronic arthritis.[10] Experimentally, by perfusion experiments,[11] a low calcium level in the perfusate leads to similar hypertrophy which is overcome by the addition of adequate amounts of calcium. Hyperphosphatemia also induces a corresponding enlargement as one would expect. Vitamin D reduces the compensatory hypertrophy induced by low calcium feedings.[12]

[1] Snyder, R. Garfield, Squires, Willard, Forster, John, Traefer, Cornelius, and Wagner, Lewis Clark, *Indust. Med.*, 1942, **11**, 295.

[2] Wagner, Lewis, *Industr. Med.*, 1942, **11**, 313.

[3] Norman, G. F., *West. J. Surg., Ob. and Gyn.*, 1938, **46**, 553.

[4] Nonidez, J. F., and Goodale, H. D., *Am. J. Anat.*, 1927, **38**, 319.

[5] Cornbleet, T., and Struck, N. C., *Arch. Dermat. and Syph.*, 1937, **35**, 188.

[6] Leriche, R., and Jung, A., *La Presse Med.*, 1938, **46**, 809.

[7] Leriche, R., *Gazette du Hospitaux*, 1936, **109**, 209; Ab. *J. A. M. A.*, 1936, **106**, 1214.

[8] Bernheim, Alice R., *J. A. M. A.*, 1933, **100**, 1001.

[9] Bernheim, Alice, and Garlock, J. H., *Ann. Surgery*, 1935, **101**, 1012.

[10] Wootton, W. T., *J. Missouri M. A.*, 1936, **33**, 129; Abs. *J. A. M. A.*, 1936, **106**, 1854.

[11] Path, Harvey M., Wallerstein, Elizabeth, and Luckhardt, Arno B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 580.

[12] Carnes, William H., Pappenheimer, Alvin M., and Stoerk, Herbert C., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 314.

It also further diminishes the size of these glands already reduced by hypophosphatemia while simultaneously raising both calcium and phosphorus levels in the blood serum.

This apparently occurs independently of the pituitary,[13,14] and the control, if any, of this organ on the parathyroids must occur through the gonads via the gonadotropic hormones.

The effects of overdosage of the parathyroid hormone or the results of tumors of these glands on the organism are well known and need only passing mention here. The demineralization of bone shafts with deformities, the calcification of soft tissues, notably kidneys, lungs, stomach, and arteries, have been described in detail elsewhere.

The present tests were carried out in 1944 and 1945 to investigate the relationship above mentioned, and are now being reported because of the increasing importance of the use of vitamin D in the treatment of arthritis and other conditions. The results obtained were used to support the clinical work undertaken by the senior author.[15]

The present study was undertaken to determine the effect of the addition of estrogens and androgens to rachitic animals administered high doses of vitamin D.

(a) The effect of the addition of the estrogen and androgen were studied in homologous and heterologous fashion.

(b) Criteria of bone-ash were employed in fixing the effect of the addition of vitamin D to a rachitic animal, and also to measure the increment, if any, due to the dosing of the estrogen or androgen, as the case may be.

Experimental. The diet used was Rachitic Diet No. 2, as advocated in the United States Pharmacopoeia Twelfth Revision, supplemented with .1 g each of riboflavin and thiamine hydrochloride per 100 lb of diet. The vitamin D administered orally to the rats was the U. S. P. Reference Oil No. 2. The hormone supplements used were testosterone propionate in sesame oil (Ciba perandren), and estradiol di-propionate in sesame oil (Ciba di-ovocylin). These supplements were injected subcutaneously. Where no hormone supplement was given the rats were injected with Wesson Oil.

All of the animals used in these experiments were of the Long-Evans strain bred from the colony maintained in this laboratory. The young animals had been raised in accordance with U. S. P. standards and were from groups which were available for U. S. P. vitamin D assays. The number of animals assigned to each group varied from 5 to 7 and the groups were adjusted to obtain approximately equal starting weights. The variability within groups due to litter was decreased by the use of isogenic segregation. Litters were chosen which had 3 to 5 animals of the same sex in order that one animal of each litter would appear in each group of a given study; for example: a triad of male rats from the same litter would be found in the control group receiving no vitamin D and no hormone, in the test group receiving only vitamin D, and in the test group receiving vitamin D and the hormone supplement. The rats were kept in individual wire-screen cages and given feed and water *ad libitum*.

In the course of these experiments bi-weekly weights of the animals were taken. At the end of the test period individual blood samples were taken as follows: An incision was made across the belly of the animal, cutting directly into the abdomen, and exposing the vena cava. The vena cava was then severed and the blood sample collected in a centrifuge tube. The chemical analysis of the blood serum was accomplished on a micro scale. Calcium was determined by the permanganate titration method of Tisdall.[16] The inorganic phosphorus was determined by precipitation of the proteins with trichloracetic acid, and a colorimetric determination was made after the reduction of the phosphomolybdic complex with stannous chloride, as advocated by Kuttner and Cohen.[17]

For bone-ash analysis the left tibiae were removed and freed of adhering tissue by buffing with cheesecloth. The bones were

[13] Albright, Fuller, *J. A. M. A.*, 1939, **112**, 2592.

[14] Carnes, Wm. H., Osebold, John, and Stoerk, Herbert C., *Am. J. Physiol.*, 1943, **139**, 188.

[15] Norman, G. F., *Geriatrics*, 1947, **2**, 24.

[16] Tisdall, F. F., *J. Biol. Chem.*, 1923, **56**, 439.

[17] Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, **75**, 517.

TABLE I.
Experiment I.
Effect of the Injection of Hormones in Vitamin D Depleted Rats Fed Various Doses of Vitamin D.

Group	Vitamin D: Daily dose, mg	Vitamin D: Total fed, mg	Hormone dosage: Medication	Hormone dosage: Total dose, mg	Net gain: Mean, g	Net gain: Std. Dev.	Tibia bone-ash %
			Males (Paired Litter Mates).				
1	0	0	Wesson Oil	0	139	± 3.87	31.12
2	6.0	210.0	" "	0	180	± 2.50	48.47
3	6.0	210.0	Perandren	17.5	148	± 4.03	46.22
7	.9	31.5	Wesson Oil	0	170	± 5.62	35.19
8	.9	31.5	Di-ovocylin	17.5	104	± 3.60	42.43
			Females (Paired Litter Mates).				
4	0	0	Wesson Oil	0	179	±3.03	36.36
5	6.0	210.0	" "	0	203	± 5.03	50.69
6	6.0	210.0	Di-ovocylin	3.5	88	± 3.35	53.53
9	.9	31.5	Wesson Oil	0	235	± 5.74	44.66
10	.9	31.5	Perandren	17.5	212	± 4.51	38.16

extracted in anhydrous isopropanol for 2 hours and then ashed by group.

On the next to the last day of the test the animals were anesthetized and individual X-rays taken of each rat. Representative X-rays are presented to show the skeletal development which resulted from the studies undertaken.

In the preliminary studies young male rats were depleted for 18-21 days on the rachitic diet and then placed on assay for 10 days under conditions prescribed in the United States Pharmacopoeia. The daily dose of vitamin D was fed orally, and hormone supplements administered subcutaneously at the start of the assay and on the fifth day of assay. Results obtained indicated that although a large difference in percent bone-ash was obtained between the control group fed no vitamin D (29.50) and the highest level of vitamin D fed 10.67 mg per day (34.61), the 10-day test period would not allow for significant changes due to the injection of the hormone. It was felt that a longer assay period would allow for more treatments and, hence, a more critical study of the role of the hormone supplements. Therefore, in the assays herein reported the "prophylactic bone-ash method" as advocated by Coward[18] was used. When the rats were weaned they were placed directly on the rachitic diet and the assay started, continuing for 5 weeks. The dose of vitamin D was one-fifth of the curative test and given twice weekly instead of daily. The hormone supplement was injected every 5 days (Sundays excluded).

Results and Discussion. As advocated by Coward,[18] the level of vitamin D to be fed in the "prophylactic bone-ash method" was one-fifth of the dose use in the curative U. S. P. assay required to give a 2-plus healing. The level of vitamin D used on assay designated as the high dose was 5 times the level required to give a 2-plus healing.

Sixty rats, ranging in age from 21-24 days, and in weight from 40-63 g, and consisting of 30 males and 30 females, were allotted into 10 groups, as shown in Table I. The 5 male groups were made up of litter-mate-brothers, and similarly the 5 female groups of litter-mate-sisters. One of the 5 groups served as the control for the 2 test groups involving the treatment with the homologous hormone, and also as the control for the other 2 groups devoted to the treatment with the heterologous hormone. The mean net gain per group, its standard error, and the percent bone-ash of the extracted tibiae are summarized in Table I.

In order to determine statistically whether or not the mean difference observed in Table I between Groups 5 and 6 was significantly different, the right tibia of each animal was prepared as in the case of the group ashings

[18] Coward, K. H., *Biological Standardisation of the Vitamins*, Baillière, Tindall and Cox, London, 1938, 121.

TABLE II.
Experiment I.
Statistical Analysis of Data.

Group 5: Females; vitamin D—high dose.
Group 6: Females; vitamin D—high dose plus Estrogen.

	Tibia Bone-ash %	
	Group 6	Group 5
	56.72	43.73
	51.83	49.09
	53.85	46.94
	53.79	49.09
	51.21	51.94
	54.04	54.43
Mean	53.57	49.20
Mean diff.		4.37
Stand. dev. of diffs.		5.14
Stand. error of mean diffs.		2.099
Critical ratio (t)		2.08

With 5 degrees of freedom the value for t when P equals .10 is 2.015, and the value found exceeds this limit.

except that now the bones were individually labeled, extracted, and ashed.

Since these groups were made up of 6 pairs of sisters the results obtained were treated according to "Student's" method of analysis for correlated data as shown in Table II.[19]

From Table I it will be seen that females tolerate depletion better than males, as indicated by the higher percent bone-ash for the control group (Group 4 vs. Group 1). This could possibly be explained by the presence of ovarian tissue.

It is obvious that the addition of a large amount of vitamin D led to an increase in body weight in both sexes, while the added medication counteracted the increase due to vitamin D.

In males the employment of estrogen significantly increased the percentage of bone-ash (Group 7 vs Group 8), while this was diminished with androgen as the medication (Group 2 vs. Group 3).

The use of the high dosage of vitamin D in the females definitely enhanced the percent bone-ash, and this was further increased with the use of the estrogen (Group 5 vs. Group 6). On the low dose of vitamin D the females were particularly sensitive to the androgen, definitely decreasing the percent bone-ash (Group 9 vs. Group 10). In this instance 2 factors apparently operate, namely, the low protection of the vitamin D dosage and the parathyroid stimulating effect of the androgen.

As shown in the statistical analysis in Table II, a significant increase in the percent bone-ash was observed when female rats given a high dose of vitamin D were concurrently treated with estrogen (Group 5 vs. Group 6).

In the first experiment with female rats the use of the estrogen definitely enhanced the percent bone-ash when given in connection with the high dose of vitamin D, and similarly showed an increase for males when the low dose of vitamin D was administered. On the other hand, the use of the androgen decreased the percent bone-ash when given in connection with the low dose of vitamin D to females, and also showed the same effect when given with the high dose of vitamin D to males.

These findings are in accord with the observations of Gardner,[20,21] who investigated the effect of estrogens and androgens on the breaking strength of femurs of mice. However, the use of rachitic animals and the employment of sex hormones in conjunction with vitamin D have not been considered. In addition, the increase in bone-ash attributed to the use of the estrogen confirms the work of Day and Follis.[22] It was proposed, therefore, to continue the study of the use of the androgen in connection with the high dose of vitamin D administered to both males and females.

Thirty-seven rats, ranging in age from 23-26 days, and in weight from 47-74 g, and consisting of 22 males and 15 females, were allotted into 6 groups, as shown in Table III. The 3 male groups were made up of litter-mate-brothers, and similarly the 3 female groups of litter-mate sisters. One of the 3 groups served as the control for the 2 test groups involving the treatment with perandren.

The 3 male groups consisted of 8 animals

19 "Student," *Metron.*, 1925, **5**, No. 3, 114.

20 Gardner, W. U., *Endocrinology*, 1943, **32**, 149.

21 Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 230.

22 Day, Harry G., and Follis, Richard H., Jr., *Endocrinology*, 1941, **28**, 83.

TABLE III.
Experiment II.
Effect of the Injection of Androgen in Vitamin D Depleted Rats Fed Various Doses of Vitamin D.

Group	Vitamin D: Daily dose, mg	Vitamin D: Total fed, mg	Androgen dosage: Medication	Androgen dosage: Total dose, mg	Net gain: Mean, g	Net gain: Std. Dev.	Tibia bone-ash, %	Analysis of serum: Calcium, Mean, mg %	Analysis of serum: Inorganic phosphorus, Mean, mg %
				Males (Paired Litter Mates).					
1	0	0	Wesson Oil	0	37.00	± 4.18	34.44	9.98	3.7
2	6	210	" "	0	37.43	± 4.70	49.03	10.57	3.6
3	6	210	Perandren	17.5	34.86	± 3.92	47.29	9.63	3.8
				Females (Paired Litter Mates).					
4	0	0	Wesson Oil	0	33.20	± 1.48	35.57	9.20	3.8
5	6	210	" "	0	29.00	± 3.85	50.60	10.80	3.1
6	6	210	Perandren	17.5	28.20	± 3.25	49.09	9.54	3.9*

* Mean of 3 samples.

in the control and seven in each of the test groups, while the 3 female groups were made up of 5 animals each. The mean net gain per group, its standard error, and the percent bone-ash of the extracted tibiae are summarized in Table III.

In the first experiment blood samples were taken, but were only used to establish the technic for handling the samples taken in this experiment. Therefore, in addition, Table III contains a summary of the analysis of the serum with respect to the calcium and inorganic phosphorus.

From Table III it will be observed that with both sexes there was a gain in weight in the animals employing vitamin D which was equal to the controls, as well as a definite increase in the percent bone-ash of the tibia. The use of androgen again diminished the percentage of bone-ash, and also reduced the net gain in weight when employed in conjunction with the vitamin D.

In the blood serum there were apparently no significant changes in the inorganic phosphorus level; however the calcium levels were depressed with the use of the androgen as the medication.

In order to determine statistically whether or not the mean difference observed in Table III between Groups 2 and 3 was significantly different, the right tibia of each animal was prepared as in the case of the group ashings except that now the bones were individually labeled, extracted, and ashed.

Since these groups were made up of 7 pairs of brothers the results obtained were treated according to "Student's" method for analysis for correlated data as shown in Table IV.[19]

TABLE IV.
Experiment II.
Statistical Analysis of Data.

Group 2: Males; vitamin D—high dose.
Group 3: Males; vitamin D—high dose plus Androgen.

	Tibia Bone-ash %	
	Group 2	Group 3
	50.35	49.29
	45.78	46.39
	48.39	46.12
	50.84	51.26
	46.50	46.63
	50.78	47.83
	47.69	44.69
Mean	48.62	47.46
Mean diff.		1.16
Stand. dev. of diff.		1.588
Stand. error of mean diff.		.600
Critical ratio (t)		1.933

With 6 degrees of freedom the value for t when P equals .10 is 1.943, and the value found approximates this limit.

The individual X-rays of the rats employed in the 2 tests were critically examined and the changes in bone structure noted. With respect to both sexes, the female sex hormone increased the density of the bone-shaft and indicated an advanced epiphyseal closure. The male sex hormone showed an opposite effect on both sexes as evidenced by a diminution of calcification of the bone-shaft and a slowed epiphyseal closure. These changes are best illustrated in representative photographs

GROUP 1
MALE
NORMAL UNCASTRATED
GROUP 2
MALE
HIGH DOSE VITAMIN D
GROUP 3
MALE
HIGH DOSE VITAMIN D PLUS ANDROGEN

FIG. 1.


44-4R
#56 - GROUP 4
FEMALE
NEGATIVE
CONTROL
44-4R
#51
GROUP 5
FEMALE
VITAMIN D
HIGH DOSE
44-4R
#54
GROUP 6
FEMALE
HIGH DOSE
VITAMIN D
PLUS ESTROGEN

FIG. 2.

of sets of litter mates taken from the assay procedures.

Conclusion. On the basis of these experiments carried out with rats, it is our conclusion, in agreement with other observers, that for animals of both sexes the female sex hormone (a) stunts the growth of young animals, (b) increases the density of the bone-shaft, (c) accelerates epiphyseal closure, (d) increases the percentage of bone-ash.

These postulates confirm the depressing effect of this hormone on the parathyroid glands; further confirmed by the greater response in this effect when female animals were used (Experiment I).

The male hormone, on the other hand, in both sexes (a) stunts the growth of the animal, (b) diminishes the density of the bone-shaft, (c) diminishes the percentage of bone-ash, (d) delays epiphyseal closure, (e) depresses slightly the blood calcium, but does not increase the serum inorganic phosphorus.

To summarize: The male sex hormone is rachitogenic; the female sex hormone is antirachitic. The use of the female hormone is suggested either alone or in conjunction with vitamin D in the treatment of either sex where there is overaction of the parathyroids.

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16219 P

Activation of Plasma Thromboplastinogen and Evidence of an Inhibitor.*

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Evidence has been presented earlier indicating that the first reaction in the coagulation of the blood is the conversion of the precursor of thromboplastin, thromboplastinogen, to the active form.[1] This is brought about by a factor in the platelets. Hemophilic platelets appear equally as active as those of normal blood. The reaction can readily be studied by the prothrombin consumption test, which consists in determining the prothrombin before and after coagulation. Typical results are presented in Table I.

Recently Milstone[2] has also obtained data showing that the thromboplastin in plasma occurs in an inactive form which he names prothrombokinase. He observed that it is activated in the presence of calcium, but he does not mention or apparently consider the platelets as a possible activation factor. From recent studies[1] it appears more likely that thromboplastin is not an enzyme but that its activation is enzymatic.

Recently it was found that the blood of a patient who developed a hemophilia-like disease (presumably as a sequela to pemphigus) contains an agent which inhibits the activation of thromboplastinogen. Significant results obtained in the study of the patient's blood are given in Table II. In addition it was found that the prothrombin time response of the patient's plasma to serial dilutions of thromboplastin is identical with that of normal plasma.

It seems clear that the impaired coagulation is not due to an anti-thromboplastin. The cause must be due either to a lack of thromboplastinogen or to failure of the latter

* This work was supported by a grant from the U. S. Public Health Service.

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1 Quick, A. J., *Am. J. Med. Sci.*, 1947, **214**, 272.

2 Milstone, J. H., *Science*, 1947, **106**, 546.

TABLE I.
Prothrombin Consumption in the Coagulation of Normal Blood, of Normal Deplateletized Plasma and of Normal Deplateletized Plasma Mixed with Hemophilic Platelets.

	One hour after coagulation	
	Prothrombin time of serum‡ sec	Prothrombin activity remaining %
Normal blood	37	10
Deplateletized* normal plasma	10½	100
Deplateletized normal plasma mixed with hemophilic platelets†	37	10

* The needle and syringe used for collecting the blood were coated with methyl-chloro silane (Dri-Film). The blood was transferred to a tube similarly coated and centrifuged at 6000 rpm in an angle centrifuge for 10 minutes.

† Washed platelets obtained from 3 cc of hemophilic plasma by means of differential centrifugation were mixed with 1 cc of deplateletized plasma.

‡ To a mixture of 0.1 cc of 0.02 M $CaCl_2$, 0.1 cc thromboplastin and 0.1 cc fresh oxalated plasma treated with $Ca_3(PO_4)_2$, 0.1 cc of serum was added.

TABLE II.
Evidence of a Substance (in the Blood of a Patient with a Hemophilia-like Condition) Which Inhibits the Activation of Thromboplastinogen.

	Coagulation time (Lee-White) min	Prothrombin activity remaining in serum 1½ hr after coagulation %
Normal blood	6	15 (32 sec)
Hemophilia-like blood	35	80+ (12 '')
Hemophilia-like blood, 1 vol., normal blood, 1 vol.	30	80+ (12 '')

to be activated. If it be a simple lack of thromboplastinogen, the addition of normal blood should restore the coagulation time approximately to normal and significantly increase the prothrombin consumption as is observed with hemophilic blood. This does not occur. In fact when one volume of the patient's blood is mixed with one volume of normal blood, the mixture has nearly as long a coagulation time as that of the patient's blood. The most obvious explanation is that this hemophilia-like blood contains an excess of a substance which inhibits the conversion of thromboplastinogen to active thromboplastin. This also explains why patients of this type are refractory to transfusions and to anti-hemophilic globulin. In uncomplicated hemophilia, the defect appears to be essentially a deficiency of thromboplastinogen, but the reports of Munro and Munro[3] and of Lawrence and Craddock[4] suggest that the disease can be complicated by the appearance in the blood of an inhibitor. It is likely that the agent is the same as the one occurring in the patient of this report.

From these results it can be concluded that a thromboplastin deficiency may arise either from a lack of thromboplastinogen in the plasma or from an agent which inhibits the platelet factor. A third possible cause may be postulated, namely, a lack of the platelet factor. All three conditions are characterized by a markedly incomplete conversion of prothrombin.

Summary. The first step in coagulation is the conversion of thromboplastinogen to active thromplastin by a platelet factor. The platelets from hemophilic blood react equally as well as those of normal blood. Evidence of a factor which inhibits the activation of thromboplastinogen has been found in the blood of a patient who has an acquired hemophilia-like condition.

[3] Munro, F. L., and Munro, M. P., *J. Clin. Invest.*, 1946, **25**, 814.

[4] Lawrence, J. S., and Craddock, C. G., *Science*, 1947, **106**, 473.

16220

Dietary Impairment of Estrogen Response in the Immature Monkey.

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We have previously reported that the genital tract of immature chicks maintained on a diet deficient in folic acid shows only a slight growth response to estrogenic hormone.[1]

The laboratory monkey (*Maccacus rhesus*) is known to require exogenous folic acid, and the characteristic features of the deficiency syndrome have been described.[2,3]

We wish to report that sexually immature monkeys which are maintained on a synthetic diet known to be deficient in folic acid fail to show the characteristic changes observed by Allen[4] and others in estrogen-treated monkeys fed a natural diet.

Ten sexually immature female monkeys weighing between 2500 and 2900 g were individually caged and fed *ad libitum* a purified diet of the following percentile composition:[5] C. P. dextrose, 73; vitamin-free casein (Smaco), 18; salt mixture, 4; corn oil, 3; cod liver oil, 2.[5] In addition, the following supplement suspended in 20 cc of water was offered each monkey daily: thiamine, 2 mg; nicotinic acid, 10 mg; riboflavin, 2 mg; pyridoxine, 2 mg; calcium pantothenate, 6 mg; choline, 100 mg; para-aminobenzoic acid, 200 mg; *i*-inositol, 200 mg; ascorbic acid, 50 mg; biotin, 50 μg; dextrose, 2 g. When a monkey failed to consume the supplement voluntarily it was administered by stomach tube.

To the diet of 2 of the monkeys 5% by weight of whole dried liver substance was added for control.

The monkeys were observed for periods ranging from 43 to 66 days. As each animal on the experimental diet developed signs of deficiency characterized by apathy, muscular weakness, diarrhea, anorexia, weight loss and leukopenia, daily subcutaneous injections of 66 μg of estradiol benzoate in 0.2 cc corn oil were administered for periods ranging from 5 to 10 days. Similar treatment was given the liver-supplemented animals after comparable periods of observation. Daily examination was made of the vaginal smear, the external genitalia, and the sexual skin.

The data are summarized in Table I. It will be seen that of 8 monkeys maintained on the deficient diet 6 failed to show any change in the external genitalia or sexual skin following estrogen treatment for 8 to 10 days. In the 2 liver-supplemented animals the characteristic external changes were readily observable by the end of the third day and were very marked by the end of the fifth day (Fig. 1 and 2). The vaginal smears in the non-responsive experimental animals showed little change following estrogen administration whereas the smears from the liver-supplemented animals showed the characteristic cornified cells.

These observations do not establish the specificity of the dietary deficiency produced

TABLE I.
Perineal Estrogen Response of "M" Deficient Monkeys.

Monkey	Days on diet	Days injected	Response
694	43	9	negative
675	53	8	"
19	60	10	"
18	60	10	"
B39	48	8	"
B18	48	8	"
B9	47	5	positive
46	48	5	"

Each monkey given 66 μg (400 R.U.) of estradiol benzoate in 0.2 cc corn oil subcutaneously daily.

1 Hertz, R., *Endocrinology*, 1945, **37**, 1.

2 Day, P. L., Langston, W. C., and Darby, W. J., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 860.

3 Day, P. L., Nims, V., and Potter, J. R., *J. B. C.*, 1945, **161**, 45.

4 Allen, E., Hisaw, F. L., and Gardner, W. V., *Sex and Internal Secretions*, 2nd Edition, 1939, Chapter VIII.

5 Weisman, H. A., Rasmussen, A. F., Elvehjem, C. A., and Clark, P. F., *J. Nutrition*, 1945, **26**, 205.

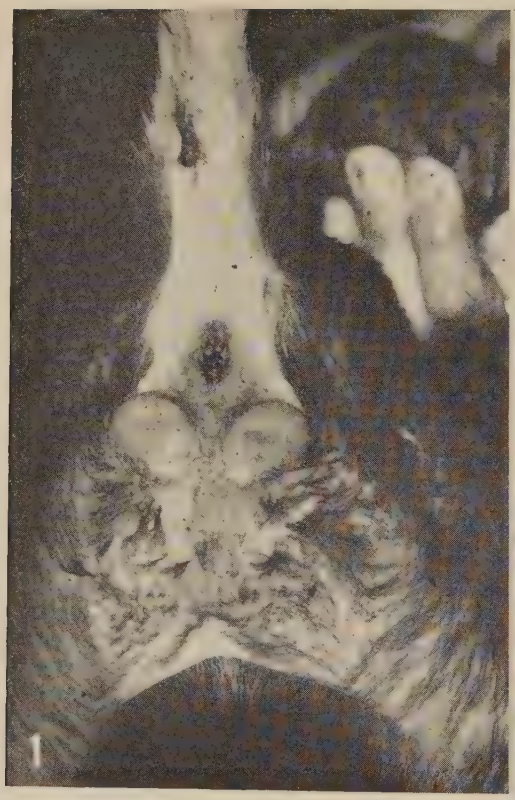

FIG. 1.
Perineum of folic acid-deficient monkey after 10 days estrogen treatment.
FIG. 2.
Perineum of control monkey after 5 days estrogen treatment.

since we have not attempted to restore the estrogen responsiveness in the folic acid deficient monkey following the administration of crystalline folic acid. However, we have observed such a restoration of estrogen response in the folic acid deficient chick following the administration of synthetic folic acid. Moreover, Day *et al.*[2] have shown that monkeys on diets comparable to that employed here show a good therapeutic response to folic acid.

Monkeys are known to show wide variability in the length of time required for the development of folic acid deficiency.[2] It is possible that the 2 animals on the deficient diet which showed a good response to estrogen were incompletely depleted of folic acid at the time they were tested, although they were not grossly distinguishable from the others.

The mechanism and the biological significance of the dependence of estrogen utilization on dietary folic acid remain obscure. The existence of this relationship in two such widely diverse forms as the chick and the monkey suggests that the phenomenon may be of physiological significance. Perhaps the well-known sex difference in erythropoiesis may depend in some way upon this interrelationship between folic acid and the estrogens. In this connection, Taber *et al.*[6] have shown that the characteristic sex difference in red count in the chick may be reversed by administration of either male or female sex hormone. Analogous findings have been presented by Vollmer *et al.*[7] for the rat.

[6] Taber, E., Davis, D., and Domm, L., *Am. J. Phys.*, 1943, **138**, 479.

[7] Vollmer, E. P., Gordon, A. S., Levenstein, I., and Charipper, A. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 409.

Summary. Of 8 sexually immature monkeys maintained on a folic acid-deficient, synthetic diet until evidence of dietary deficiency appeared, 6 failed to show the characteristic normal response to estrogen administration. Two control monkeys fed the same diet plus liver and 2 of the apparently deficient animals showed typical estrogen response to similar hormone treatment.

16221 P

Renal Resistance in "Essential" Hypertension. Relation to the Effect of Sympathectomy on Blood Pressure.*

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Blood pressure and renal dynamics were studied preoperatively in 13 hypertensive patients subjected to extensive sympathectomy in 1940-42.[1,2] These observations were repeated on 12 of these cases at intervals extending up to 8 to 40 months after operation. Current analysis of these data suggests that the preoperative renal resistance may indicate with reasonable accuracy whether a significant reduction in blood pressure will follow the operative procedure. While renal resistances were also calculated by the method of Lamport,[3] a value for "crude" renal resistance is satisfactory. The "crude" renal resistance is defined as $\frac{\text{Blood Pressure}}{\text{Renal Blood Flow}} \times 100$. The arithmetical mean of systolic and diastolic brachial arterial pressure determined by sphygmomanometer, is taken as the blood pressure; and the diodrast or para-amino hippurate clearance of blood in cc per minute per 1.73 square meters of body surface is considered to represent renal blood flow. The resistance value obtained was above normal in all cases. It was less than 22 in 5 cases with definite postoperative reduction in blood pressure and was 22 or more in 7 cases with little or no reduction in blood pressure. The means of the postoperative diastolic blood pressure in these two groups were 88.8 and 120.3 mm Hg, respectively, the difference between these being significant (Fig. 1), when subjected to Fisher's method for the comparison of small samples.[4] Diastolic pressures were averaged for each patient during periods of hospitalization for the studies. The pressures taken during the renal tests for each patient tend to be slightly higher than the corresponding hospital averages. Using either "test" diastolic, or ½ (systolic plus diastolic) pressure values, the difference in the means of post-operative pressure in the two groups arbitrarily divided according to resistance, remains significant ($t = 5.41, 4.49$).

It is therefore unlikely that chance alone would produce the results obtained in this grouping. However, the data of our series alone is inadequate to prove that the difference in resistance is the only responsible factor. Several other factors may be involved. Four of the patients where blood pressure was markedly reduced by operation were the 4 youngest in the series. If we divide our patients according to age, a significant difference in the mean of postoperative pressure is noted when this division is made at or under 35 years (Fig. 1). The

* Aided by a grant from the Douglas Smith Foundation.

1 Adams, W., Alving, A. S., Sandiford, I., Grimson, K. S., and Scott, C., *Am. J. Physiol.* (Proceedings), 1941, **133**, 190.

2 Grimson, K. S., Adams, W., Alving, A. S., and Landowne, M., in preparation.

3 Lamport, H., *J. Clin. Invest.*, 1943, **22**, 461.

4 Fisher, R. A., *Statistical Methods for Research Workers*, 10th edition, G. E. Stechert, New York, p. 122, 1946.

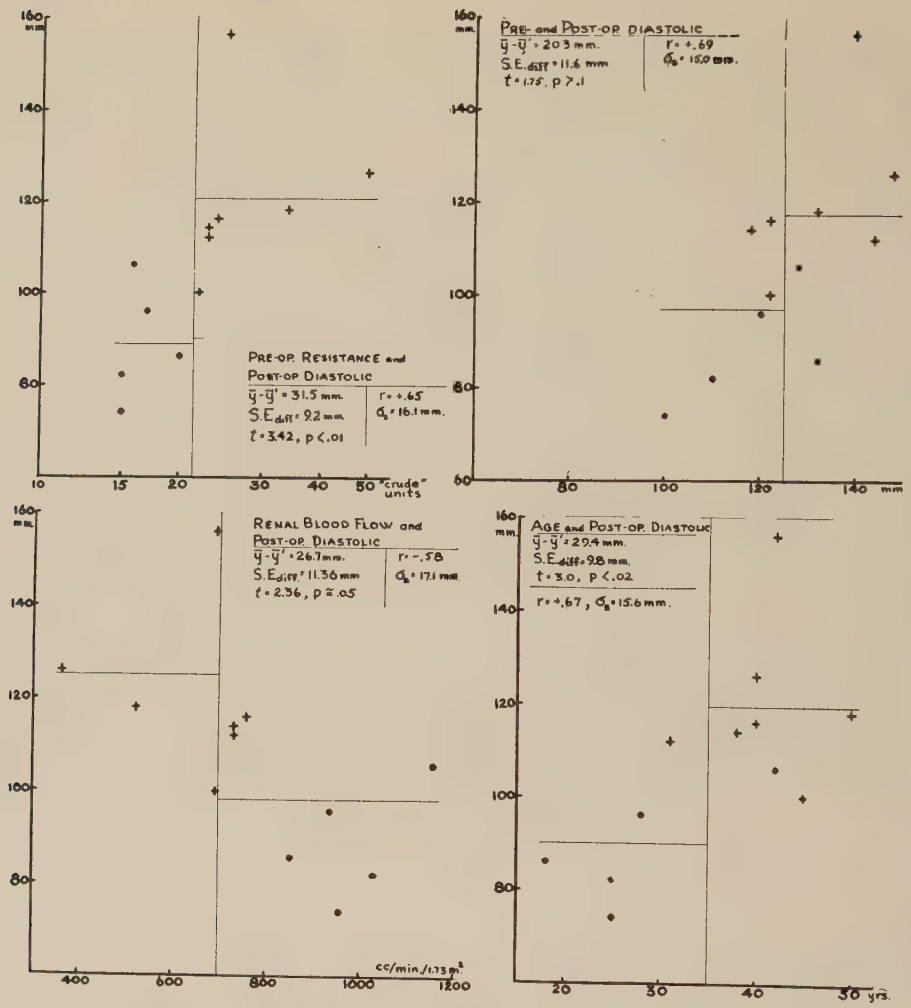

FIG. 1.

Scatter diagrams from data of 12 hypertensive patients subjected to sympathectomy. The averaged post-operative diastolic pressure in mm Hg (ordinates) is related to each of 4 pre-operative measurements. r indicates the first order coefficients for the data as plotted, and σ_s the standard error of the estimate. The means of ordinate values greater and less than arbitrary vertical divisions are indicated by horizontal lines. The difference of the means (y–y'), the standard error of this difference ($S.E._{diff.}$), their ratio (t) and the probability value (p) are shown.

average preoperative "crude" resistance of the patients 35 years of age or younger was lower than the average of the patients over 35 years of age, but the difference has little significance ($p > 0.05$). Analysis of the component values used to derive "crude" resistance indicates that a grouping of cases on the basis of preoperative diastolic pressure level reveals no significant differences in results. Division according to preoperative renal blood flows at or below the value of 549 cc per minute selected by Foà *et al.*,[5] or even at 700 cc per minute, does not result in a significant difference in the postoperative diastolic means. Only if the cases are divided at a renal blood flow of 800 cc per minute, are significant differences manifest. At this level the grouping is identical to that obtained by division at a "crude" resistance of 22 units.

[5] Foà, P. P., Woods, W. W., Peet, M. M., and Foà, N. L., *Arch. Intern. Med.*, 1943, **71**, 357.

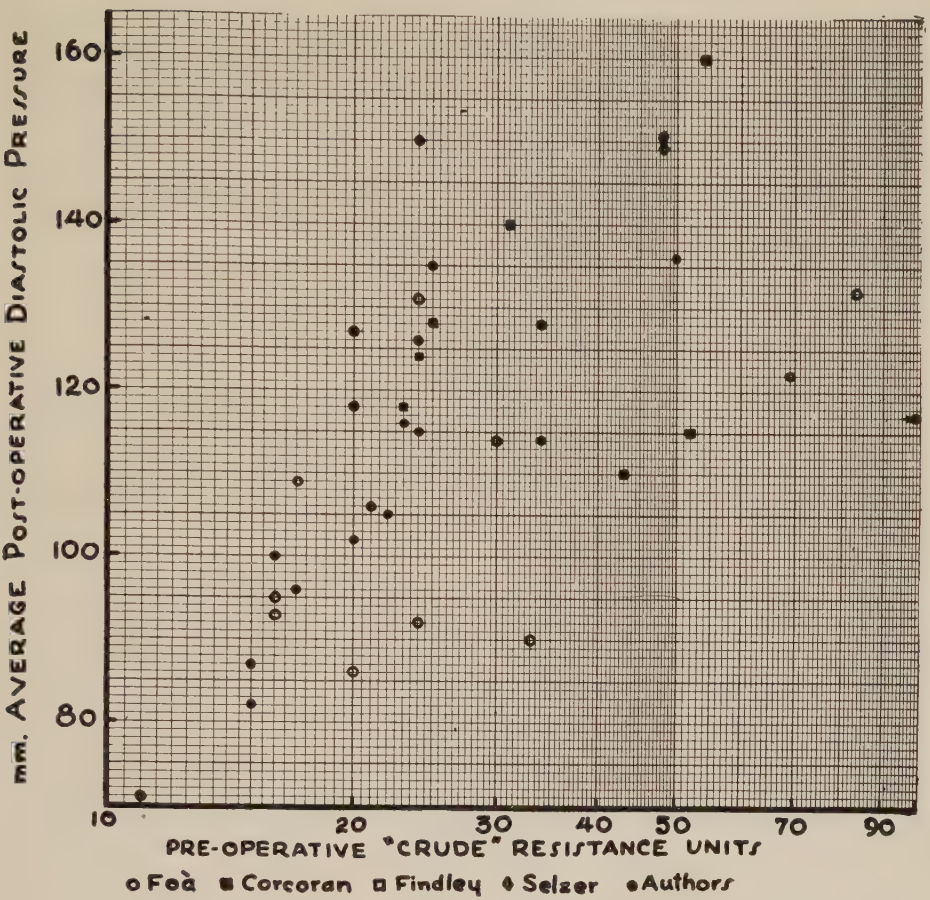

FIG. 2.

Composite scatter diagram relating preoperative renal resistance in "crude" units to the average postoperative diastolic pressure in mm Hg of 38 patients with hypertension subjected to sympathectomy. Twenty-six cases are calculated from data in the literature.[5-8]

Fig. 1 also indicates the zero order coefficients of correlation (r) and the standard error of the estimate (σ_s) of the relation of postoperative pressure to each of the 4 parameters mentioned, viz., log "crude" preoperative resistance, preoperative diastolic pressure, preoperative renal blood flow, and age. All show good correlation but with a wide scatter.

Fig. 2 indicates the relation between the postoperative diastolic pressure and the preoperative "crude" resistance, again for the present series (black dots) and for the calculated values in 26 additional cases reported in the literature.[5,6,7,8] If these 26 cases are similarly divided into 2 groups on the basis of whether their calculated preoperative "crude" resistances are below 22 units, or are 22 or more; the differences of the means of their postoperative diastolic pressures is also highly significant ($y - y' = 25$ mm, $p < 0.01$). No significant differences in postoperative diastolic means are revealed by groupings using age, preoperative diastolic pressures, or renal blood flows. If divided at a renal blood flow of 549 cc per minute, p is greater than 0.1, and, if the higher level of 700 cc per minute is used, the likelihood that chance explains the difference is almost as great

6 Corcoran, A. C., and Page, I. H., *Arch. Surg.*, 1941, **42**, 1072.

7 Selzer, A., and Friedman, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 429.

8 Findley, T., Clinton, E., and Edwards, J. C., *Surgery*, 1942, **12**, 64.

($y - y' = 20.2$ mm, $p > 0.05$). Only 4 cases had preoperative renal blood flows above 800 cc per minute. Resistance values were calculated from the publications of these authors, using pressures at the time of the tests wherever designated by them, and without modification of data. Where hematocrit was not given, a value of 0.43 was assumed.

Conclusion. A relation of the preoperative renal resistance to the postoperative blood pressure has been demonstrated in patients with hypertension subjected to sympathectomy. A complete test of the reliability of this relation in predicting the result of sympathectomy requires additional data.

16222

Antagonism by Cellular Extracts of Effects of Respiratory Poisons on Onion Roots and Sea Urchin Eggs.

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Aqueous yeast extract has been shown to antagonize the deleterious effects of potassium cyanide, basic phenylmercuric nitrate and sodium azide upon respiration[1,2,3,4] and of basic phenylmercuric nitrate on the growth[1,5,6] of unicellular organisms and fragments of mammalian tissues. Study of the antagonism of yeast extract against the effects of these respiratory poisons has now been extended to intact multicellular structures, namely, the roots of the onion and the eggs of the sea urchin.

Onion Roots. Onions (*Allium cepa*) of approximately 5 cm in diameter, purchased in market, were germinated over water in the dark at room temperature. When the roots were approximately 4-7 cm in length they were reduced in number to 10-25 of uniform appearance. The normal rate of growth was 1 cm to 1.5 cm per day. The poisons employed were basic phenylmercuric nitrate (PMN) in concentrations of 1:200,000 to 1:24,000 (7.5×10^{-6} to 6.3×10^{-5} M); potassium cyanide, 0.4% (0.06 M); mercuric chloride, 1:72,000 (5.1×10^{-5} M), and urethane, 3 and 5% (0.34 M and 0.56 M).

The crude yeast extract, in dilutions of 0.8 to 2.5%, was an alcoholic extract corresponding to Sample A as described by Cook, Kreke, and Nutini.[7] In a few experiments beef spleen extract[8] was used in concentrations of 0.4 and 0.2% with concentrations of 1.6×10^{-5} PMN. The onion roots were immersed about half their total length in the test solutions for 1 hour and were thoroughly rinsed before replacement in tap water. The lethal dose of the poison was established as the amount which would lead to the death of all of the roots as a consequence of 1 hour of exposure. On surviving roots, enlarged tips, if they developed, usually did so in the 24 hours following exposure to the test solutions.

1 Cook, E. S., and Kreke, C. W., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 222.

2 Cook, E. S., Kreke, C. W., Eilert, Sr. M. R., and Sawyer, M. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 210.

3 Kreke, C. W., and Suter, Sr. M. St. A., *J. Biol. Chem.*, 1945, **160**, 105.

4 Kreke, C. W., and Suter, Sr. M. St. A., *Studies Inst. Divi Thomae*, 1945, **4**, 85.

5 Cook, E. S., and Kreke, C. W., *Nature*, 1940, **146**, 688.

6 Thomas, G. W., Fardon, J. C., Baker, S. L., and Cook, E. S., *J. Am. Pharm. Assn., Sci. Ed.*, 1945, **34**, 143.

7 Cook, E. S., Kreke, C. W., and Nutini, L. G., *Studies Inst. Divi Thomae*, 1938, **2**, 23.

8 Staff, I. D. T., *Studies Inst. Divi Thomae*, 1947, **5**, 55.

TABLE I.
Gross Effects on Onion Roots After One Hour of Exposure to Respiratory Poisons and Yeast Extract.

Concentrations		Dead roots, %	Terminal swellings, %	Growth arrest, hr	Total growth 4 days, cm
Poison	Yeast extract, %				
0	0	0	0	0	4-6
		Basic Phenylmercuric Nitrate.			
1:200,000	—	0	100	24	2-3
(7.5 x 10-6 M)	1.0	0	100	24	2-3
1:96,000	—	100	—	—	—
(1.6 x 10-5 M)	0.8	0	100	24	2-3
1:70,000	—	100	—	—	—
(2.1 x 10-5 M)	1.0	0	100	12-24	2-4
1:50,000	1.2	0	76	12-24	2-3
(3.0 x 10-5 M)	2.5	0	100	48*	1-1.5
1:48,000†	—	100	—	—	—
(3.1 x 10-5 M)	0.5	0	100	12-48	2-3
1:30,000	—	100	—	—	—
(5.0 x 10-5 M)	0.5	100	—	—	—
	1.0	80	20	72	1
1:24,000	—	100	—	—	—
(6.3 x 10-5 M)	1.0	100	—	—	—
	2.0	21	0	Indef.	?
	2.5	0	100	12-72	0.2-3.5
		Potassium Cyanide.			
0.4%	—	100	—	—	—
(0.06 M)	1.0	0	0	Slight	3.5-4
		Mercuric Chloride.			
1:72.000	—	100	—	—	—
(5.1 x 10-5 M)	1.0	0	0	Slight	4-4.5‡

* On'y 50% resumed growth.
† Only 30 minutes exposure.
‡ Secondary roots developed, after 5 days, from root tips and along entire immersed length.

The results are summarized in Table I. When the effects of the test solutions were sublethal, growth by elongation, in many instances was arrested in the succeeding 24-hour interval. During this time small swellings developed just behind the meristematic tip which were similar in appearance to those produced by exposure to colchicine and to various organic salts.[9] Growth was usually resumed at a reduced rate on the second day and distal to the terminal swelling (Table I).

Yeast extract in no way modified the effects of a sublethal concentration of PMN, but prevented the lethal action of concentrations of the poison as high as 6.3 x 10^{-5} M, with the subsequent production of terminal swellings. In one experiment (PMN, 3 x 10^{-5} M, and yeast extract, 2.5%) only 50% of the roots resumed growth within 4 days. To determine if this was in part due to the high concentration of the yeast extract, onion roots were exposed to a 2.5% concentration of the extract alone. In the next 4 days their average growth was only 5 cm while that of control roots on the same bulb which had been in water throughout, was 6 cm. By the 4th day the daily increment was equivalent to that of the control roots.

Against lethal amounts of KCN and $HgCl_2$, a 1% concentration of yeast extract entirely protected the roots with no swellings or irregularities in form developing.

The action of urethane on onion roots differed from that of the PMN, KCN, and $HgCl_2$. A 5% solution (0.56 M) acting for 1 hour was not lethal. Growth was arrested for 48 hours but no swellings developed. Exposure for as long as 6 hours to a 3% (0.34 M) solution was not lethal at the end of that time and elongation continued for the next 24 hours, but was arrested on the second day, during which time terminal swellings developed. By the 4th day the tiny meristematic tip distal to the swollen zone was dead.

9 Levan, A., *Nature*, 1945, **156**, 751.

Twenty-four hours after exposure to 3% urethane and 1% yeast extract the roots were flaccid and dead in a zone 1 cm in length immediately behind the meristematic tip; no terminal swellings developed during the interval.

Two experiments with aqueous beef spleen extract in concentrations of 0.4 and 0.2% were made using 1:96,000 PMN (1.6×10^{-5} M), for an hour of exposure. With 0.4% beef spleen extract none of the roots was dead at the end of the exposure period. Growth was arrested for 24 hours, during which time 2 of the 12 roots developed small terminal swellings. Very slight growth (1-2 cm) resulted during the 5 days following exposure. With 0.2% spleen extract, 4 roots were dead at the end of 48 hours; the remaining 10 continued to grow very slowly, 2 developing swollen tips and the others becoming curved and irregular in form.

Sea Urchin Embryos. Fertilized eggs in the 2- to 4-cell stage of the sea urchin (*Tripneustes esculentus*) were exposed for 1 hour to basic PMN and to mixtures of PMN and yeast extract and of PMN and beef spleen extract. Suspensions of fertilized eggs were prepared according to Just's method.[10] About 10 ml of thick egg suspensions containing a few hundred eggs were diluted to 30 ml with fresh sea water and used for each experiment. In control experiments the developing eggs were ciliated motile blastulae within 20 hours. The concentrations of PMN used were 1:144,000 (1×10^{-5} M), 1:1.5 million (1×10^{-6} M), and 1:2 million (7.5×10^{-6} M); 1:3.2 million (4.7×10^{-7} M) and 1:4 million (3.8×10^{-7} M). The crude yeast extract was diluted to 0.5, 0.8, and 1.0%.

With all of the concentrations of PMN, except the most dilute solution, 95 to 100% of the eggs were dead at the end of 20 hours after exposure to the poison for 1 hour. Some of the eggs (43, 30 and 95%) resumed growth after removal from the more concentrated solutions of PMN (1×10^{-5} M, 1×10^{-6} M, 7.5×10^{-7} M), but only 2% of those in 1×10^{-6} M reached the blastula stage and became motile. Most of the embryos treated divided but once more. Many of the embryos were anomalous in form and some of the cells cytolyzed. In the weakest dilution (3.8×10^{-7} M) the eggs were undamaged after 1 hour of exposure to the poison, resuming a normal rate of growth and development except for the slowing of the rate of absorption of the yolk.

Yeast extract (0.8%) did not protect the eggs against the lethal action of PMN 1×10^{-5} M, although a larger percentage resumed growth for a short period, after removal to seawater, than was the case with eggs exposed to PMN of the same concentration alone. None of the cells was cytolyzed. All of the eggs exposed to PMN 1×10^{-6} M and 1% yeast extract resumed growth, and half reached the motile blastula stage as contrasted with the 30% resuming growth, and 2% reaching the blastula stage after exposure to the poison alone. Mortality at 20 hours with yeast extract and the poison was 50% as compared to 95% without extract. There were also fewer anomalous forms and less cytolysis among the eggs exposed to the mixture of poison and yeast extract than among those exposed to the poison alone. At a PMN concentration of 4.7×10^{-7} M, yeast extract (0.8%) completely overcame the deleterious effects of PMN.

Evidence of a retarding action of the yeast extract itself lies in the observation that there was a more nearly normal rate of yolk absorption after exposure to PMN 3.8×10^{-7} M with 0.5% extract than with 1.0% of extract. Beef spleen extract at 0.8% concentration was less effective than yeast extract in antagonizing the deleterious effects of PMN on sea urchin eggs.

Discussion. It has been shown that, while basic phenylmercuric nitrate inhibits enzymes dependent upon the –SH group for their activity, it also inhibits iron porphyrin enzymes such as catalase and cytochrome oxidase;[11,12,13] azide and cyanide likewise inhibit

[10] Just, E. E., *Basic Methods on Eggs of Marine Animals*, Philadelphia, P. Blakiston's Son and Co., 1939.

[11] Cook, E. S., Kreke, C. W., McDevitt, Sr. M. of L., and Bartlett, Sr. M. D., *J. Biol. Chem.*, 1946, **162**, 43.

[12] Cook, E. S., and Perisutti, G., *J. Biol. Chem.*, 1947, **167**, 827.

the iron porphyrin enzymes.[14] Yeast extract has accelerated the activity of cytochrome oxidase and offset the effects of PMN (Cook and Kreke, unpublished) and poisons specific for this enzyme.[3,4] Moreover, yeast extract fails to antagonize poisoning of the respiration of yeast and mammalian tissue by urethane which inhibits the dehydrogenases.[3,4] In the present work, the action of urethane on onion roots differed from that of cyanide and PMN in 2 respects, in that (a) the meristematic cells were first killed and (b) this effect could not be offset with the concentrations of yeast extract used.

The effects of respiratory poisons and yeast extract on multicellular structures appear to resemble those described for unicellular organisms and mammalian tissue fragments. Cytologic studies are now in progress to determine if the toxic action is also evident in intracellular modifications. In these experiments reaction of the extracts with the poisons can not be excluded nor can possible direct effects of the extract on the enzyme systems or on the cell membranes. Further experiments will be undertaken to obtain information on these points.

Summary. The lethal and sub-lethal effects of basic phenylmercuric nitrate, potassium cyanide and mercuric chloride on onion roots and sea urchin embryos can be antagonized by yeast and beef spleen extract. These observations are in agreement with reports that yeast extract can offset the action of respiratory poisons on some enzyme systems.

[13] Cook, E. S., Kreke, C. W., and Walsh, Sr. T. M., *J. Biol. Chem.*, 1946, **162**, 51.

[14] McElroy, W. D., *Quart. Rev. Biol.*, 1947, **22**, 25.

(Continued from page ii)